FEMS Microbiology Letters

AUTHOR INDEX VOLUME 56

Adler, L., see Trollmo, C. (56) 321
Allardet-Servent, A., see Segondy, M. (56) 177
Amann, R., Ludwig, W., Laubinger, W., Dimroth, P. and Schleifer, K.H.
Cloning and sequencing of the gene encoding the beta subunit of the sodium ion translocating ATP synthase of *Propionigenium modestum* (56) 253
André, L., see Trollmo, C. (56) 321
Anraku, Y., see Yamato, I. (56) 21
Aparicio, J.F., see De los Reyes-Gavilán, C.G. (56) 301
Arbuthnott, J.P., see Huang, J. (56) 109
Avraham, Y., Yashphe, J. and Grossowicz, N.
Thymidine phosphorylase and uridine phosphorylase of *Lactobacillus casei* (56) 29

Barbés, C., see De los Reyes-Gavilán, C.G. (56) 301
Barbotin, J.N., see Sayadi, S. (56) 307
Barnum, S.R., see Smoker, J.A. (56) 119
Bénichou, J.-C., see Fréhel, C. (56) 225
Beppu, T., see Tagami, H. (56) 161
Berg, W.D., see Peterson, J.W. (56) 139
Berry, F., see Sayadi, S. (56) 307
Bhatnagar, N., see Falah, A.M.S. (56) 89
Bhatnagar, R., see Falah, A.M.S. (56) 89
Blomberg, A., see Trollmo, C. (56) 321
Borriello, S.P., see Kamiya, S. (56) 331
Bosch, A., see Tartera, C. (56) 313
Bothe, H., see Häger, K.-P. (56) 189
Bouvet, O.M.M. and Grimont, P.A.D.
Evolutionary divergence of enterobacterial genes coding for the glucose phosphotransferase system components (56) 145
Boylan, M., see Twohig, J. (56) 327
Brehm, J.K., see Oultram, J.D. (56) 83

C., G., see Hien, N.T. (56) 337
Caravano, R., see Segondy, M. (56) 177
Cazzulo, J.J., Nowicki, C., Santome, J.A., Wernstedt, C. and Hellman, U.
Amino acid composition and N-terminal sequence of the NADP-linked glutamate dehydrogenase from *Trypanosoma cruzi* (56) 215
Cervantes, C. and Ohtake, H.
Plasmid-determined resistance to chromate in *Pseudomonas aeruginosa* (56) 173
Cevenini, R., Donati, M. and La Placa, M.
Effects of penicillin on the synthesis of membrane proteins of *Chlamydia trachomatis* LGV2 serotype (56) 41
Chakraborty, R.N., see Patel, H.N. (56) 131
Chernyak, B.V., see Dmitriev, O.Y. (56) 79
Chew, L.C.K., Tacon, W.C.A. and Cole, J.A.
Effect of growth conditions on the rate of loss of the plasmid pAT153 from continuous cultures of *Escherichia coli* HB101 (56) 101
Christiansen, L., see Diderichsen, B. (56) 53
Cianciotto, N.P., Long, R., Eisenstein, B.I. and Engleberg, N.C.
Site-specific mutagenesis in *Legionella pneumophila* by allelic exchange using counterselectable ColE1 vectors (56) 203
Clark, D.P., see Reams, S.G. (56) 231
Cole, J.A., see Chew, L.C.K. (56) 101
Coso, O.A., see Rubinstein, C.P. (56) 67
Courvalin, P., see Sougakoff, W. (56) 343
Cowan, D., see Lambert, E. (56) 157
Crabbe, M.J.C.
The effect of thiols and the Ca^{2+} ionophore A23817 on growth and antibiotic production in *Cephalosporium acremonium* (56) 71

Daniel, R.M., see Patel, B.K.C. (56) 285
De los Reyes-Gavilán, C.G., Aparicio, J.F., Barbés, C., Hardisson, C. and Sánchez, J.
Adsorption capability determines phage plaque size on *Streptomyces antibioticus* producing endodeoxyribonuclease (56) 301
Dembek, G. and Lingens, F.
Isolation and characterization of a meta-cleavage product in the degradation of quinaldic acid by *Azotobacter sp.* (56) 261
Desai, S.B., see Patel, H.N. (56) 131
Diderichsen, B. and Christiansen, L.
Cloning of a maltogenic alpha-amylase from *Bacillus stearothermophilus* (56) 53
Dimroth, P., see Amann, R. (56) 253
Dion, M., see Zealey, G. (56) 123
Dmitriev, O.Y. and Chernyak, B.V.
The properties and structure of the membrane ATPase from *Vibrio alginolyticus* (56) 79
Donachie, W. and Gilmour, N.J.L.
Sheep antibody response to cell wall antigens expressed in vivo by *Pasteurella haemolytica* serotype A2 (56) 271
Donati, M., see Cevenini, R. (56) 41
Droffner, M.L., see Yamamoto, N. (56) 209

Earhart, C.F., see Elkins, M.F. (56) 35

Egan, A.F., see Shay, B.J. (56) 183
Eisenstein, B.I., see Cianciotto, N.P. (56) 203
Elkins, M.F. and Earhart, C.F.
An *Escherichia coli* enterobactin cluster gene with sequence homology to *trpE* and *pabB* (56) 35
Engleberg, N.C., see Cianciotto, N.P. (56) 203
Ericsson, L.D., see Stack, R.J. (56) 1

Fairbrother, J.M., Lallier, R., Leblanc, L., Jacques, M. and Larivière, S.
Production and purification of *Escherichia coli* fimbrial antigen F165 (56) 247
Falah, A.M.S., Bhatnagar, R., Bhatnagar, N., Singh, Y., Sidhu, G.S., Murthy, P.S. and Venkitasubramanian, T.A.
On the presence of calmodulin-like protein in mycobacteria (56) 89
Flores, E. and Muro-Pastor, M.I.
Uptake of glutamine and glutamate by the dinitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120 (56) 127
Fréhel, C., Rastogi, N., Bénichou, J.-C. and Ryter, A.
Do test tube-grown pathogenic mycobacteria possess a protective capsule? (56) 225
Fukaya, M., see Tagami, H. (56) 161

Galeazzi, L., Grilli, G., Turchetti, G., Groppa, G. and Giunta, S.
High reactivity of the chlorpromazine/hydrogen peroxide test with cytochrome oxidase-positive bacteria (56) 317
Gil, I., see Gómez, E. (56) 47
Gilmour, N.J.L., see Donachie, W. (56) 271
Giunta, S., see Galeazzi, L. (56) 317
Goldhar, J., see Grünberg, J. (56) 241
Gómez, E., Melgar, M.M., Pérez Silva, G., Portolés, A. and Gil, I.
Exocellular products from *Bifidobacterium adolescentis* as immunomodifiers in the lymphoproliferative responses of mouse splenocytes (56) 47
Goussard, S., see Sougakoff, W. (56) 343
Grant, W.D., see Hamamoto, T. (56) 221
Grilli, G., see Galeazzi, L. (56) 317
Grimont, P.A.D., see Bouvet, O.M.M. (56) 145
Groman, N.B., see Serwold-Davis, T.M. (56) 7
Groppa, G., see Galeazzi, L. (56) 317
Grossowicz, N., see Avraham, Y. (56) 29
Gruber, K., Tanahashi, H., Tsuboi, A., Tsukagoshi, N. and Udaka, S.
Comparative study on the cell wall structure of protein-producing *Bacillus brevis* (56) 113
Grünberg, J., Perry, R., Hoschützky, H., Jann, B., Jann, K. and Goldhar, J.
Nonfimbrial blood group N-specific adhesin (NFA-3) from *Escherichia coli* O20 : KX104 : H^-, causing systemic infection (56) 241

Hacker, J., see Muñoa, F. (56) 167
Hadley, W.M., see McDowell, T.D. (56) 151
Häger, K.-P., Hundeshagen, B. and Bothe, H.
The demonstration of reversed respiration in intact cells of chemolithoautotrophically grown *Bradyrhizobium japonicum* (56) 189
Hamamoto, T., Takashina, T., Grant, W.D. and Horikoshi, K.
Asymmetric cell division of a triangular halophilic archaebacterium (56) 221
Hardisson, C., see De los Reyes-Gavilán, C.G. (56) 301
Hellman, U., see Cazzulo, J.J. (56) 215
Hien, N.T., Kerby, N.W., C., G., Machray, , Rowell, P. and Stewart, W.D.P.
Expression of glutamine synthetase in mutant strains of the cyanobacterium *Anabaena variabilis* which liberate ammonia (56) 337
Hiraishi, A.
Bicarbonate-stimulated dark fermentative growth of a phototrophic purple nonsulfur bacterium (56) 199
Honda, T., see Kongmuang, U. (56) 105
Horikoshi, K., see Hamamoto, T. (56) 221
Horinouchi, S., see Tagami, H. (56) 161
Hoschützky, H., see Grünberg, J. (56) 241
Huang, J., Smyth, C.J., Kennedy, N.P., Arbuthnott, J.P. and Napoleon Keeling, P.W.
Haemagglutinating activity of *Campylobacter pylori* (56) 109
Hudson, J.A., see Patel, B.K.C. (56) 285
Hundeshagen, B., see Häger, K.-P. (56) 189

Izumi, Y., Kanzaki, H., Morita, S., Kato, N. and Yamada, H.
Dichlorophenolindophenol-linked formate dehydrogenase of the methanol-utilizing *Mycobacterium gastri* MB19 (56) 277

Jacques, M., see Fairbrother, J.M. (56) 247
Jann, B., see Grünberg, J. (56) 241
Jann, K., see Grünberg, J. (56) 241
Javor, G.T. and Kim, H.
A simple method for screening porphyrin secretion by colonies of *Escherichia coli* (56) 195
Jerez, C.A.
The heat shock response in meso- and thermoacidophilic chemolithotrophic bacteria (56) 289
Jofre, J., see Tartera, C. (56) 313
Juárez, A., see Muñoa, F. (56) 167

Kamiya, S., Reed, P.J. and Borriello, S.P.
Analysis of purity of *Clostridium difficile* toxin A derived by affinity chromatography on immobilized bovine thyroglobulin (56) 331
Kanzaki, H., see Izumi, Y. (56) 277
Kato, N., see Izumi, Y. (56) 277
Kawamura, Y., see Tagami, H. (56) 161
Kempf, B. and Träger, L.
Production of monoclonal antibodies against 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (56) 237
Kennedy, N.P., see Huang, J. (56) 109
Kerby, N.W., see Hien, N.T. (56) 337

Kim, H., see Javor, G.T. (56) 195
Klein, M., see Zealey, G. (56) 123
Kok, J., see Suvorov, A. (56) 95
Kongmuang, U., Honda, T. and Miwatani, T.
Effect of nicking on Shiga-like toxin I of enterohaemorrhagic *Escherichia coli* (56) 105
Kurland, C.G., see Mikkola, R. (56) 265

Lac, B., see Willison, J.C. (56) 61
Lallier, R., see Fairbrother, J.M. (56) 247
Lambert, E., Cowan, D. and Piper, P.W.
Detection of sequences homologous to the highly-conserved HSP70 gene of eukaryotes in thermophilic eubacteria and archaebacteria (56) 157
La Placa, M., see Cevenini, R. (56) 41
Larivière, S., see Fairbrother, J.M. (56) 247
Laubinger, W., see Amann, R. (56) 253
Leblanc, L., see Fairbrother, J.M. (56) 247
Lingens, F., see Dembek, G. (56) 261
Long, R., see Cianciotto, N.P. (56) 203
Loosmore, S., see Zealey, G. (56) 123
Loughlin, M., see Oultram, J.D. (56) 83
Ludwig, W., see Amann, R. (56) 253

Machray, , see Hien, N.T. (56) 337
Mahadevan, A., see Vasudevan, N. (56) 349
Massad, G., Simpson, L.M. and Oliver, J.D.
Isolation and characterization of hemolysin mutants of *Vibrio vulnificus* (56) 295
McDowell, T.D., Reed, K.E. and Hadley, W.M.
Accumulation of ppGpp in three streptococci during periods of amino acid starvation (56) 151
McKay, A.M.
A plate assay method for the detection of fungal polygalacturonase secretion (56) 354
Melgar, M.M., see Gómez, E. (56) 47
Mikkola, R. and Kurland, C.G.
Media dependence of translational mutant phenotype (56) 265
Minton, N.P., see Oultram, J.D. (56) 83
Miwatani, T., see Kongmuang, U. (56) 105
Moratinos, L.C., see Rubinstein, C.P. (56) 67
Morgan, H.W., see Patel, B.K.C. (56) 285
Morita, S., see Izumi, Y. (56) 277
Muñoa, F., Hacker, J. and Juárez, A.
Characterization of a chromosomal mutant that blocks hemolysin excretion in *Escherichia coli* (56) 167
Murakami, H., see Yamato, I. (56) 21
Muro-Pastor, M.I., see Flores, E. (56) 127
Murthy, P.S., see Falah, A.M.S. (56) 89

Nakamura, H., see Yamato, I. (56) 21
Napoleon Keeling, P.W., see Huang, J. (56) 109
Nasri, M., see Sayadi, S. (56) 307
Nowicki, C., see Cazzulo, J.J. (56) 215

Ochoa, L.G., see Peterson, J.W. (56) 139
Ohtake, H., see Cervantes, C. (56) 173
Okumura, H., see Tagami, H. (56) 161
Oliver, J.D., see Massad, G. (56) 295
Oultram, J.D., Loughlin, M., Swinfield, T.-J., Brehm, J.K., Thompson, D.E. and Minton, N.P.
Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation (56) 83

Patel, B.K.C., Hudson, J.A., Morgan, H.W. and Daniel, R.M.
Non-diauxic fermentation of acid hydrolysed pine by a strain of *Clostridium thermohydrosulfuricum* isolated from a New Zealand hot spring (56) 285
Patel, H.N., Chakraborty, R.N. and Desai, S.B.
Isolation and partial characterization of phenolate siderophore from *Rhizobium leguminosarum* IARI 102 (56) 131
Pérez Silva, G., see Gómez, E. (56) 47
Perry, R., see Grünberg, J. (56) 241
Peterson, J.B.
The interaction of metronidazole with electron carriers of the *Azotobacter vinelandii* respiratory particle fraction (56) 135
Peterson, J.W., Ochoa, L.G. and Berg, W.D.
Inhibitory effect of ibuprofen on the cholera toxin-induced cyclic AMP response in Chinese hamster ovary cells (56) 139
Piper, P.W., see Lambert, E. (56) 157
Portolés, A., see Gómez, E. (56) 47

Ramuz, M., see Segondy, M. (56) 177
Rastogi, N., see Fréhel, C. (56) 225
Reams, S.G. and Clark, D.P.
Glucose repression of anaerobic genes of *Escherichia coli* is independent of cyclic AMP (56) 231
Reed, K.E., see McDowell, T.D. (56) 151
Reed, P.J., see Kamiya, S. (56) 331
Rogers, H.J., see Wright, G. (56) 15
Rogers, P.J., see Shay, B.J. (56) 183
Rowell, P., see Hien, N.T. (56) 337
Rubinstein, C.P., Moratinos, L.C., Coso, O.A. and Sánchez-Rivas, C.
Improvements in the transformation of *Bacillus subtilis* protoplasts with plasmid DNA (56) 67
Ryter, A., see Fréhel, C. (56) 225

Sánchez, J., see De los Reyes-Gavilán, C.G. (56) 301
Sánchez-Rivas, C., see Rubinstein, C.P. (56) 67
Santome, J.A., see Cazzulo, J.J. (56) 215
Sayadi, S., Berry, F., Nasri, M., Barbotin, J.N. and Thomas, D.
Increased stability of pBR322-related plasmids in *Escherichia coli* W3101 grown in carrageenan gel beads (56) 307
Schleifer, K.H., see Amann, R. (56) 253
Segondy, M., Allardet-Servent, A., Caravano, R. and Ramuz, M.
Common physical map of four *Brucella* bacteriophage genomes (56) 177
Serwold-Davis, T.M. and Groman, N.B.

Identification of a methylase gene for erythromycin resistance within the sequence of a spontaneously deleting fragment of *Corynebacterium diphtheriae* plasmid pNG2 (56) 7
Shay, B.J., Egan, A.F., Wright, M. and Rogers, P.J.
Cysteine metabolism in an isolate of *Lactobacillus sake*: Plasmid composition and cysteine transport (56) 183
Sidhu, G.S., see Falah, A.M.S. (56) 89
Simpson, L.M., see Massad, G. (56) 295
Singh, S.
Regulation of urea-uptake in the cyanobacterium *Anabaena doliolum* (56) 281
Singh, Y., see Falah, A.M.S. (56) 89
Smoker, J.A. and Barnum, S.R.
Rapid small-scale DNA isolation from filamentous cyanobacteria (56) 119
Smyth, C.J., see Huang, J. (56) 109
Smyth, C.J., see Twohig, J. (56) 327
Sougakoff, W., Goussard, S. and Courvalin, P.
The TEM-3 β-lactamase, which hydrolyzes broad-spectrum cephalosporins, is derived from the TEM-2 penicillinase by two amino acid substitutions (56) 343
Stack, R.J. and Ericsson, L.D.
Methods for the identification and analysis of L-altrose in bacterial polysaccharides (56) 1
Stewart, W.D.P., see Hien, N.T. (56) 337
Suvorov, A., Kok, J. and Venema, G.
Transformation of group A streptococci by electroporation (56) 95
Swinfield, T.-J., see Oultram, J.D. (56) 83

Tacon, W.C.A., see Chew, L.C.K. (56) 101
Tagami, H., Tayama, K., Tohyama, T., Fukaya, M., Okumura, H., Kawamura, Y., Horinouchi, S. and Beppu, T.
Purification and properties of a site-specific restriction endonuclease *Aaa*I from *Acetobacter aceti* subsp. *aceti* No. 1023 (56) 161
Takashina, T., see Hamamoto, T. (56) 221
Tanahashi, H., see Gruber, K. (56) 113
Tartera, C., Bosch, A. and Jofre, J.
The inactivation of bacteriophages infecting *Bacteroides fragilis* by chlorine treatment and UV-irradiation (56) 313
Tayama, K., see Tagami, H. (56) 161
Thomas, D., see Sayadi, S. (56) 307
Thompson, D.E., see Oultram, J.D. (56) 83
Tohyama, T., see Tagami, H. (56) 161
Träger, L., see Kempf, B. (56) 237
Trollmo, C., André, L., Blomberg, A. and Adler, L.
Physiological overlap between osmotolerance and thermotolerance in *Saccharomyces cerevisiae* (56) 321
Tsuboi, A., see Gruber, K. (56) 113
Tsukagoshi, N., see Gruber, K. (56) 113
Turchetti, G., see Galeazzi, L. (56) 317
Twohig, J., Boylan, M. and Smyth, C.J.
Expression of CS fimbriae in *Escherichia coli* strains of human and animal origin belonging to O-serovar 6, K-serovar 15 or H-serovar 16 (56) 327

Udaka, S., see Gruber, K. (56) 113

Vasudevan, N. and Mahadevan, A.
Degradation of *p*-benzyloxyphenol by *Acinetobacter* sp. (56) 349
Venema, G., see Suvorov, A. (56) 95
Venkitasubramanian, T.A., see Falah, A.M.S. (56) 89

Wernstedt, C., see Cazzulo, J.J. (56) 215
Willison, J.C. and Lac, B.
Behaviour of Nif$^-$ mutants of *Rhodobacter capsulatus* in photosynthetic, ammonia-limited chemostat cultures (56) 61
Wright, G. and Rogers, H.J.
Spherical and rod shaped protoplasts from β-lactam antibiotic treated cultures of *Bacillus subtilis* (56) 15
Wright, M., see Shay, B.J. (56) 183

Yacoob, R., see Zealey, G. (56) 123
Yamada, H., see Izumi, Y. (56) 277
Yamamoto, N. and Droffner, M.L.
Anaerobic expression of the *gyrA* gene as demonstrated by β-galactosidase activity in *gyrA*::Mu-*lac* fusion derivatives of *Escherichia coli* (56) 209
Yamato, I., Nakamura, H., Murakami, H. and Anraku, Y.
Mapping and disruption of the *cybB* gene coding for cytochrome b_{561} in *Escherichia coli* (56) 21
Yashphe, J., see Avraham, Y. (56) 29

Zealey, G., Dion, M., Loosmore, S., Yacoob, R. and Klein, M.
High frequency transformation of *Bordetella* by electroporation (56) 123

FEMS Microbiology Letters

SUBJECT INDEX VOLUME 56

gyrA
DNA gyrase; Strict aerobic growth; Anaerobiosis; Gene fusion; Nalidixic acid resistance (Yamamoto, N. (56) 209)

Acetobacter
Restriction endonuclease (Tagami, H. (56) 161)

***Acinetobacter* sp.**
Lignin model substance; Degradation (Vasudevan, N. (56) 349)

Acquired osmotolerance
Conditioning; Acquired thermotolerance; Physiological overlap; *sn*-Glycerol 3-P dehydrogenase; Heat shock protein; *Saccharomyces cerevisiae* (Trollmo, C. (56) 321)

Acquired thermotolerance
Conditioning; Acquired osmotolerance; Physiological overlap; *sn*-Glycerol 3-P dehydrogenase; Heat shock protein; *Saccharomyces cerevisiae* (Trollmo, C. (56) 321)

Actin
Penicillin; Cephalosporin; *Cephalosporium acremonium*; Blastospore; Cytochalasins; Cysteine; Glutathione; Reductases (Crabbe, M.J.C. (56) 71)

Adenosine triphosphatase
Vibrio alginolyticus; Cell membrane (Dmitriev, O.Y. (56) 79)

Adhesin
Antral gastritis; Duodenal ulcer; Pathogenesis; Virulence factor (Huang, J. (56) 109)

Adsorption
Phage adsorption; *Streptomyces antibioticus*; Endodeoxyribonuclease (De los Reyes-Gavilán, C.G. (56) 301)

Affinity chromatography
Clostridium difficile toxin A; Bovine thyroglobulin (Kamiya, S. (56) 331)

Alditol acetates
Butyrivibrio fibrisolvens; L-altrose; 1,6-anhydroaltrose; Extracellular polysaccharides; (−)-2-Octanol (Stack, R.J. (56) 1)

Allelic exchange
Legionella pneumophila; Conjugal DNA transfer; Counterselectable plasmids; U937 cells (Cianciotto, N.P. (56) 203)

Amino acid composition
Trypanosoma cruzi; Glutamate dehydrogenase; NAD; NADP; Purification; N-terminal sequence (Cazzulo, J.J. (56) 215)

Amino acid transport
(Flores, E. (56) 127)

Ammonia production
Anabaena variabilis; Glutamine synthetase; mRNA; Mutant strain (Hien, N.T. (56) 337)

Anabaena doliolum
Urea-uptake; Cyanobacterium; Regulation (Singh, S. (56) 281)

Anabaena variabilis
Ammonia production; Glutamine synthetase; mRNA; Mutant strain (Hien, N.T. (56) 337)

Anaerobe
Thermophile; Carbohydrate utilisation (Patel, B.K.C. (56) 285)

Anaerobic
Catabolite repression; Gene fusion; Beta-galactosidase (Reams, S.G. (56) 231)

Anaerobiosis
DNA gyrase; Strict aerobic growth; Gene fusion; Nalidixic acid resistance; *gyrA* (Yamamoto, N. (56) 209)

1,6-anhydroaltrose
Butyrivibrio fibrisolvens; L-altrose; Extracellular polysaccharides; (−)-2-Octanol; Alditol acetates (Stack, R.J. (56) 1)

Antigen
Sheep antibody; *Pasteurella haemolytica*; SDS PAGE; Immunoblotting (Donachie, W. (56) 271)

Antral gastritis
Duodenal ulcer; Pathogenesis; Virulence factor; Adhesin (Huang, J. (56) 109)

ATP synthase
β-subunit gene; Nucleotide sequence; *Propionigenium modestum* (Amann, R. (56) 253)

Azotobacter sp.
Degradation of quinaldic acid; Meta-cleavage product; 2-oxo-3-(4′-hydroxy-2′-oxo-3′,4′-en-butyrate)-pyridine-6-carboxylic acid; Chemical mutagenesis (Dembek, G. (56) 261)

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Azotobacter vinelandii
Metronidazole; Flavin; Cytochrome (Peterson, J.B. (56) 135)

Bacillus brevis
Cell wall; S-layer (Gruber, K. (56) 113)

Bacillus subtilis
Protoplast; β-Lactam antibiotic (Wright, G. (56) 15)

Maltogenic amylase; Cloning; Sequence (Diderichsen, B. (56) 53)

Protoplast; Transformation; Plasmid (Rubinstein, C.P. (56) 67)

Bacterial peroxidase detection
Chlorpromazine; Cytochrome oxidase-positive bacteria (Galeazzi, L. (56) 317)

Bacteriophages
Disinfection; UV-irradiation; Chlorine; Tap water; Sewage; *Bacteroides fragilis* (Tartera, C. (56) 313)

Bacteroides fragilis
Disinfection; UV-irradiation; Chlorine; Bacteriophages; Tap water; Sewage (Tartera, C. (56) 313)

Beta-galactosidase
Catabolite repression; Gene fusion; Anaerobic (Reams, S.G. (56) 231)

Bifidobacteria
Blastogenesis (Gómez, E. (56) 47)

β-lactamase
Klebsiella pneumoniae; Nucleotide sequence; Mutation (Sougakoff, W. (56) 343)

Blastogenesis
Bifidobacteria (Gómez, E. (56) 47)

Blastospore
Penicillin; Cephalosporin; *Cephalosporium acremonium*; Actin; Cytochalasins; Cysteine; Glutathione; Reductases (Crabbe, M.J.C. (56) 71)

Blood group N-specific adhesin
Escherichia coli haemagglutinin; Nonfimbrial adhesin (Grünberg, J. (56) 241)

Bordetella
Transformation; Electroporation; Electric field pulses (Zealey, G. (56) 123)

Bovine thyroglobulin
Clostridium difficile toxin A; Affinity chromatography (Kamiya, S. (56) 331)

Bradyrhizobium japonicum
Reversed respiratory electron transport; Chemolithoautotrophy; Reductive pentose phosphate cycle; Nitrogen fixation (Häger, K.-P. (56) 189)

pBR322-related plasmid
Continuous culture; Immobilized cell; Plasmid stability; Plasmid copy number (Sayadi, S. (56) 307)

Brucella phages
DNA; Restriction map (Segondy, M. (56) 177)

β-subunit gene
ATP synthase; Nucleotide sequence; *Propionigenium modestum* (Amann, R. (56) 253)

Butyrivibrio fibrisolvens
L-altrose; 1,6-anhydroaltrose; Extracellular polysaccharides; (−)-2-Octanol; Alditol acetates (Stack, R.J. (56) 1)

Calcium binding protein
Calmodulin; Mycobacteria (Falah, A.M.S. (56) 89)

Calmodulin
Calcium binding protein; Mycobacteria (Falah, A.M.S. (56) 89)

Capsule
Mycobacteria; Electron microscopy (Fréhel, C. (56) 225)

Carbohydrate utilisation
Thermophile; Anaerobe (Patel, B.K.C. (56) 285)

Catabolite repression
Gene fusion; Beta-galactosidase; Anaerobic (Reams, S.G. (56) 231)

Cell division
Triangular bacterium; Cell plate; Halobacterium; Cinematography (Hamamoto, T. (56) 221)

Cell membrane
Vibrio alginolyticus; Adenosine triphosphatase (Dmitriev, O.Y. (56) 79)

Cell plate
Triangular bacterium; Cell division; Halobacterium; Cinematography (Hamamoto, T. (56) 221)

Cell wall
Bacillus brevis; S-layer (Gruber, K. (56) 113)

Cephalosporin
Penicillin; *Cephalosporium acremonium*; Blastospore; Actin; Cytochalasins; Cysteine; Glutathione; Reductases (Crabbe, M.J.C. (56) 71)

Cephalosporium acremonium
Penicillin; Cephalosporin; Blastospore; Actin; Cytochalasins; Cysteine; Glutathione; Reductases (Crabbe, M.J.C. (56) 71)

Chemical mutagenesis
Degradation of quinaldic acid; Meta-cleavage product; 2-oxo-3-(4′-hydroxy-2′-oxo-3′,4′-en-butyrate)-pyridine-6-carboxylic acid; *Azotobacter sp.* (Dembek, G. (56) 261)

Chemolithoautotrophy
Reversed respiratory electron transport; Reductive pentose phosphate cycle; Nitrogen fixation; *Bradyrhizobium japonicum* (Häger, K.-P. (56) 189)

Chemostat culture
Escherichia coli; Plasmid; Genetic stability (Chew, L.C.K. (56) 101)

Chlamydia
Penicillin; Outer membrane proteins; Protein expression (Cevenini, R. (56) 41)

Chlorine
Disinfection; UV-irradiation; Bacteriophages; Tap water; Sewage; *Bacteroides fragilis* (Tartera, C. (56) 313)

Chlorpromazine
Bacterial peroxidase detection; Cytochrome oxidase-positive bacteria (Galeazzi, L. (56) 317)

Cholera toxin
Ibuprofen (Peterson, J.W. (56) 139)

Chorismate
Enterobactin; Isochorismate synthase; Iron regulation (Elkins, M.F. (56) 35)

Cinematography
Triangular bacterium; Cell plate; Cell division; Halobacterium (Hamamoto, T. (56) 221)

Cloning
Maltogenic amylase; Sequence; *Bacillus subtilis* (Diderichsen, B. (56) 53)

Cloning vector
Clostridium acetobutylicum; Transformation; Electroporation (Oultram, J.D. (56) 83)

Clostridium acetobutylicum
Transformation; Cloning vector; Electroporation (Oultram, J.D. (56) 83)

***Clostridium difficile* toxin A**
Affinity chromatography; Bovine thyroglobulin (Kamiya, S. (56) 331)

Colonization factor antigen II
CS3 fimbriae; Host restricted expression of CS fimbriae; Enterotoxigenic *Escherichia coli* (Twohig, J. (56) 327)

Conditioning
Acquired osmotolerance; Acquired thermotolerance; Physiological overlap; *sn*-Glycerol 3-P dehydrogenase; Heat shock protein; *Saccharomyces cerevisiae* (Trollmo, C. (56) 321)

Conjugal DNA transfer
Legionella pneumophila; Counterselectable plasmids; Allelic exchange; U937 cells (Cianciotto, N.P. (56) 203)

Continuous culture
Nif^- mutants; *Rhodobacter capsulatus*; H_2 production (Willison, J.C. (56) 61)

pBR322-related plasmid; Immobilized cell; Plasmid stability; Plasmid copy number (Sayadi, S. (56) 307)

Corynebacterium diphtheria
Macrolide-lincosamide-streptogramin B resistance; Erythromycin resistance; Methylase gene; Spontaneous deletion; *R*-plasmid (Serwold-Davis, T.M. (56) 7)

Counterselectable plasmids
Legionella pneumophila; Conjugal DNA transfer; Allelic exchange; U937 cells (Cianciotto, N.P. (56) 203)

CrO_4^{2-} resistance
P. aeruginosa; Plasmid (Cervantes, C. (56) 173)

CS3 fimbriae
Colonization factor antigen II; Host restricted expression of CS fimbriae; Enterotoxigenic *Escherichia coli* (Twohig, J. (56) 327)

Cyanobacteria
DNA isolation; Plasmid (Smoker, J.A. (56) 119)

Cyanobacterium
Anabaena doliolum; Urea-uptake; Regulation (Singh, S. (56) 281)

Cysteine
Penicillin; Cephalosporin; *Cephalosporium acremonium*; Blastospore; Actin; Cytochalasins; Glutathione; Reductases (Crabbe, M.J.C. (56) 71)

Lactic acid; *Lactobacillus sake*; Plasmid composition (Shay, B.J. (56) 183)

Cytochalasins
Penicillin; Cephalosporin; *Cephalosporium acremonium*; Blastospore; Actin; Cysteine; Glutathione; Reductases (Crabbe, M.J.C. (56) 71)

Cytochrome
Azotobacter vinelandii; Metronidazole; Flavin (Peterson, J.B. (56) 135)

Cytochrome b_{561}
Gene, cybB; *Escherichia coli* (Yamato, I. (56) 21)

Cytochrome oxidase-positive bacteria
Chlorpromazine; Bacterial peroxidase detection (Galeazzi, L. (56) 317)

Cytotoxic activity
Vero toxin; Nicked and unnicked Shiga-like toxin (Kongmuang, U. (56) 105)

Cytotoxin
Virulence; *Vibrio vulnificus* (Massad, G. (56) 295)

Degradation
Acinetobacter sp.; Lignin model substance (Vasudevan, N. (56) 349)

Degradation of quinaldic acid
Meta-cleavage product; 2-oxo-3-(4′-hydroxy-2′-oxo-3′,4′-en-butyrate)-pyridine-6-carboxylic acid; Chemical mutagenesis; *Azotobacter sp.* (Dembek, G. (56) 261)

2,3-DHBA
Rhizobium leguminosarum IARI 102; Siderophore; Iron (Patel, H.N. (56) 131)

Disinfection
UV-irradiation; Chlorine; Bacteriophages; Tap water; Sewage; *Bacteroides fragilis* (Tartera, C. (56) 313)

DNA
Brucella phages; Restriction map (Segondy, M. (56) 177)

DNA gyrase
Strict aerobic growth; Anaerobiosis; Gene fusion; Nalidixic acid resistance; *gyrA* (Yamamoto, N. (56) 209)

DNA hybridization
Enterobacteriaceae; Glucose transport (Bouvet, O.M.M. (56) 145)

DNA isolation
Cyanobacteria; Plasmid (Smoker, J.A. (56) 119)

Duodenal ulcer
Antral gastritis; Pathogenesis; Virulence factor; Adhesin (Huang, J. (56) 109)

Electric field pulses
Bordetella; Transformation; Electroporation (Zealey, G. (56) 123)

Electron microscopy
Mycobacteria; Capsule (Fréhel, C. (56) 225)

Electroporation
Clostridium acetobutylicum; Transformation; Cloning vector (Oultram, J.D. (56) 83)

Streptococcus pyogenes; Transformation; Plasmid (Suvorov, A. (56) 95)

Bordetella; Transformation; Electric field pulses (Zealey, G. (56) 123)

Endodeoxyribonuclease
Phage adsorption; Adsorption; *Streptomyces antibioticus* (De los Reyes-Gavilán, C.G. (56) 301)

Enterobacteriaceae
Glucose transport; DNA hybridization (Bouvet, O.M.M. (56) 145)

Enterobactin
Chorismate; Isochorismate synthase; Iron regulation (Elkins, M.F. (56) 35)

Enterotoxigenic *Escherichia coli*
Colonization factor antigen II; CS3 fimbriae; Host restricted expression of CS fimbriae (Twohig, J. (56) 327)

Erythromycin resistance
Corynebacterium diphtheria; Macrolide-lincosamide-streptogramin B resistance; Methylase gene; Spontaneous deletion; *R*-plasmid (Serwold-Davis, T.M. (56) 7)

Escherichia coli
Gene, cybB; Cytochrome b_{561} (Yamato, I. (56) 21)

Chemostat culture; Plasmid; Genetic stability (Chew, L.C.K. (56) 101)

Hemolysin excretion; Mutant (Muñoa, F. (56) 167)

Fimbrial antigen F165; Production; Purification (Fairbrother, J.M. (56) 247)

Ribosomal mutant (Mikkola, R. (56) 265)

***Escherichia coli* haemagglutinin**
Nonfimbrial adhesin; Blood group N-specific adhesin (Grünberg, J. (56) 241)

Ethanol
Heat shock response; *Sulfolobus acidocaldarius*; *Thiobacillus ferrooxidans*; Thermotolerance; Protein synthesis (Jerez, C.A. (56) 289)

Evolutionary conservation / eubacteria / archaebacteria
Yeast HSP70 gene; Thermophiles (Lambert, E. (56) 157)

Extracellular polysaccharides
Butyrivibrio fibrisolvens; L-altrose; 1,6-anhydroaltrose; (−)-2-Octanol; Alditol acetates (Stack, R.J. (56) 1)

Fermentation
Phototrophic bacteria; Fumarate reduction (Hiraishi, A. (56) 199)

Fimbrial antigen F165
Escherichia coli; Production; Purification (Fairbrother, J.M. (56) 247)

Flavin
Azotobacter vinelandii; Metronidazole; Cytochrome (Peterson, J.B. (56) 135)

Formate dehydrogenase
Methylotroph; *Mycobacterium gastri* (Izumi, Y. (56) 277)

Fumarate reduction
Fermentation; Phototrophic bacteria (Hiraishi, A. (56) 199)

Fungal polygalacturonase
Plate assay; Ruthenium red (McKay, A.M. (56) 354)

Gene, cybB
Cytochrome b_{561}; *Escherichia coli* (Yamato, I. (56) 21)

Gene fusion
DNA gyrase; Strict aerobic growth; Anaerobiosis; Nalidixic acid resistance; *gyrA* (Yamamoto, N. (56) 209)

Catabolite repression; Beta-galactosidase; Anaerobic (Reams, S.G. (56) 231)

Genetic stability
Escherichia coli; Chemostat culture; Plasmid (Chew, L.C.K. (56) 101)

Glucose transport
Enterobacteriaceae; DNA hybridization (Bouvet, O.M.M. (56) 145)

Glutamate dehydrogenase
Trypanosoma cruzi; NAD; NADP; Purification; Amino acid composition; N-terminal sequence (Cazzulo, J.J. (56) 215)

Glutamine synthetase
Ammonia production; *Anabaena variabilis*; mRNA; Mutant strain (Hien, N.T. (56) 337)

Glutathione
Penicillin; Cephalosporin; *Cephalosporium acremonium*; Blastospore; Actin; Cytochalasins; Cysteine; Reductases (Crabbe, M.J.C. (56) 71)

***sn*-Glycerol 3-P dehydrogenase**
Conditioning; Acquired osmotolerance; Acquired thermotolerance; Physiological overlap; Heat shock protein; *Saccharomyces cerevisiae* (Trollmo, C. (56) 321)

Halobacterium
Triangular bacterium; Cell plate; Cell division; Cinematography (Hamamoto, T. (56) 221)

Heat shock protein
Conditioning; Acquired osmotolerance; Acquired thermotolerance; Physiological overlap; *sn*-Glycerol 3-P dehydrogenase; *Saccharomyces cerevisiae* (Trollmo, C. (56) 321)

Heat shock response
Sulfolobus acidocaldarius; *Thiobacillus ferrooxidans*; Thermotolerance; Protein synthesis; Ethanol (Jerez, C.A. (56) 289)

Hemolysin excretion
Escherichia coli; Mutant (Muñoa, F. (56) 167)

Host restricted expression of CS fimbriae
Colonization factor antigen II; CS3 fimbriae; Enterotoxigenic *Escherichia coli* (Twohig, J. (56) 327)

H_2 production
Nif$^-$ mutants; *Rhodobacter capsulatus*; Continuous culture (Willison, J.C. (56) 61)

Ibuprofen
Cholera toxin (Peterson, J.W. (56) 139)

Immobilized cell
pBR322-related plasmid; Continuous culture; Plasmid stability; Plasmid copy number (Sayadi, S. (56) 307)

Immunoblotting
Sheep antibody; *Pasteurella haemolytica*; Antigen; SDS PAGE (Donachie, W. (56) 271)

Iron
Rhizobium leguminosarum IARI 102; Siderophore; 2,3-DHBA (Patel, H.N. (56) 131)

Iron regulation
Enterobactin; Chorismate; Isochorismate synthase (Elkins, M.F. (56) 35)

Isochorismate synthase
Enterobactin; Chorismate; Iron regulation (Elkins, M.F. (56) 35)

Klebsiella pneumoniae
β-lactamase; Nucleotide sequence; Mutation (Sougakoff, W. (56) 343)

β-Lactam antibiotic
Bacillus subtilis; Protoplast (Wright, G. (56) 15)

Lactic acid
Cysteine; *Lactobacillus sake*; Plasmid composition (Shay, B.J. (56) 183)

Lactobacillus casei
Thymidine phosphorylase; Uridine phosphorylase (Avraham, Y. (56) 29)

Lactobacillus sake
Cysteine; Lactic acid; Plasmid composition (Shay, B.J. (56) 183)

L-altrose
Butyrivibrio fibrisolvens; 1,6-anhydroaltrose; Extracellular polysaccharides; (−)-2-Octanol; Alditol acetates (Stack, R.J. (56) 1)

Legionella pneumophila
Conjugal DNA transfer; Counterselectable plasmids; Allelic exchange; U937 cells (Cianciotto, N.P. (56) 203)

Lignin model substance
Acinetobacter sp.; Degradation (Vasudevan, N. (56) 349)

Macrolide-lincosamide-streptogramin B resistance
Corynebacterium diphtheria; Erythromycin resistance; Methylase gene; Spontaneous deletion; *R*-plasmid (Serwold-Davis, T.M. (56) 7)

Maltogenic amylase
Cloning; Sequence; *Bacillus subtilis* (Diderichsen, B. (56) 53)

Meta-cleavage product
Degradation of quinaldic acid; 2-oxo-3-(4′-hydroxy-2′-oxo-3′,4′-en-butyrate)-pyridine-6-carboxylic acid; Chemical mutagenesis; *Azotobacter sp.* (Dembek, G. (56) 261)

Methylase gene
Corynebacterium diphtheria; Macrolide-lincosamide-streptogramin B resistance; Erythromycin resistance; Spontaneous deletion; *R*-plasmid (Serwold-Davis, T.M. (56) 7)

Methylotroph
Formate dehydrogenase; *Mycobacterium gastri* (Izumi, Y. (56) 277)

Metronidazole
Azotobacter vinelandii; Flavin; Cytochrome (Peterson, J.B. (56) 135)

Monoclonal antibodies
Steroid-transforming enzyme; *Streptomyces hydrogenans* (Kempf, B. (56) 237)

Mutant
Hemolysin excretion; *Escherichia coli* (Muñoa, F. (56) 167)

Mutant selection
Porphyrin metabolism (Javor, G.T. (56) 195)

Mutant strain
Ammonia production; *Anabaena variabilis*; Glutamine synthetase; mRNA (Hien, N.T. (56) 337)

Mutation
β-lactamase; *Klebsiella pneumoniae*; Nucleotide sequence (Sougakoff, W. (56) 343)

Mycobacteria
Calmodulin; Calcium binding protein (Falah, A.M.S. (56) 89)

Capsule; Electron microscopy (Fréhel, C. (56) 225)

Mycobacterium gastri
Formate dehydrogenase; Methylotroph (Izumi, Y. (56) 277)

NAD
Trypanosoma cruzi; Glutamate dehydrogenase; NADP; Purification; Amino acid composition; N-terminal sequence (Cazzulo, J.J. (56) 215)

NADP
Trypanosoma cruzi; Glutamate dehydrogenase; NAD; Purification; Amino acid composition; N-terminal sequence (Cazzulo, J.J. (56) 215)

Nalidixic acid resistance
DNA gyrase; Strict aerobic growth; Anaerobiosis; Gene fusion; *gyrA* (Yamamoto, N. (56) 209)

Nicked and unnicked Shiga-like toxin
Vero toxin; Cytotoxic activity (Kongmuang, U. (56) 105)

Nif ⁻ mutants
Rhodobacter capsulatus; Continuous culture; H_2 production (Willison, J.C. (56) 61)

Nitrogen fixation
Reversed respiratory electron transport; Chemolithoautotrophy; Reductive pentose phosphate cycle; *Bradyrhizobium japonicum* (Häger, K.-P. (56) 189)

Nonfimbrial adhesin
Escherichia coli haemagglutinin; Blood group N-specific adhesin (Grünberg, J. (56) 241)

N-terminal sequence
Trypanosoma cruzi; Glutamate dehydrogenase; NAD; NADP; Purification; Amino acid composition (Cazzulo, J.J. (56) 215)

Nucleotide sequence
ATP synthase; β-subunit gene; *Propionigenium modestum* (Amann, R. (56) 253)

β-lactamase; *Klebsiella pneumoniae*; Mutation (Sougakoff, W. (56) 343)

(−)-2-Octanol
Butyrivibrio fibrisolvens; L-altrose; 1,6-anhydroaltrose; Extracellular polysaccharides; Alditol acetates (Stack, R.J. (56) 1)

Outer membrane proteins
Penicillin; *Chlamydia*; Protein expression (Cevenini, R. (56) 41)

2-oxo-3-(4′-hydroxy-2′-oxo-3′,4′-en-butyrate)-pyridine-6-carboxylic acid
Degradation of quinaldic acid; Meta-cleavage product; Chemical mutagenesis; *Azotobacter sp.* (Dembek, G. (56) 261)

P. aeruginosa
CrO_4^{2-} resistance; Plasmid (Cervantes, C. (56) 173)

Pasteurella haemolytica
Sheep antibody; Antigen; SDS PAGE; Immunoblotting (Donachie, W. (56) 271)

Pathogenesis
Antral gastritis; Duodenal ulcer; Virulence factor; Adhesin (Huang, J. (56) 109)

Penicillin
Outer membrane proteins; *Chlamydia*; Protein expression (Cevenini, R. (56) 41)

Cephalosporin; *Cephalosporium acremonium*; Blastospore; Actin; Cytochalasins; Cysteine; Glutathione; Reductases (Crabbe, M.J.C. (56) 71)

Phage adsorption
Adsorption; *Streptomyces antibioticus*; Endodeoxyribonuclease (De los Reyes-Gavilán, C.G. (56) 301)

Phototrophic bacteria
Fermentation; Fumarate reduction (Hiraishi, A. (56) 199)

Physiological overlap
Conditioning; Acquired osmotolerance; Acquired thermotolerance; *sn*-Glycerol 3-P dehydrogenase; Heat shock protein; *Saccharomyces cerevisiae* (Trollmo, C. (56) 321)

Plasmid
Bacillus subtilis; Protoplast; Transformation (Rubinstein, C.P. (56) 67)

Streptococcus pyogenes; Transformation; Electroporation (Suvorov, A. (56) 95)

Escherichia coli; Chemostat culture; Genetic stability (Chew, L.C.K. (56) 101)

Cyanobacteria; DNA isolation (Smoker, J.A. (56) 119)

CrO_4^{2-} resistance; *P. aeruginosa* (Cervantes, C. (56) 173)

Plasmid composition
Cysteine; Lactic acid; *Lactobacillus sake* (Shay, B.J. (56) 183)

Plasmid copy number
pBR322-related plasmid; Continuous culture; Immobilized cell; Plasmid stability (Sayadi, S. (56) 307)

Plasmid stability
pBR322-related plasmid; Continuous culture; Immobilized cell; Plasmid copy number (Sayadi, S. (56) 307)

Plate assay
Fungal polygalacturonase; Ruthenium red (McKay, A.M. (56) 354)

Porphyrin metabolism
Mutant selection (Javor, G.T. (56) 195)

Production
Escherichia coli; Fimbrial antigen F165; Purification (Fairbrother, J.M. (56) 247)

Propionigenium modestum
ATP synthase; β-subunit gene; Nucleotide sequence (Amann, R. (56) 253)

Protein expression
Penicillin; Outer membrane proteins; *Chlamydia* (Cevenini, R. (56) 41)

Protein synthesis
Heat shock response; *Sulfolobus acidocaldarius*; *Thiobacillus ferrooxidans*; Thermotolerance; Ethanol (Jerez, C.A. (56) 289)

Protoplast
Bacillus subtilis; β-Lactam antibiotic (Wright, G. (56) 15)

Bacillus subtilis; Transformation; Plasmid (Rubinstein, C.P. (56) 67)

Purification
Trypanosoma cruzi; Glutamate dehydrogenase; NAD; NADP; Amino acid composition; N-terminal sequence (Cazzulo, J.J. (56) 215)

Escherichia coli; Fimbrial antigen F165; Production (Fairbrother, J.M. (56) 247)

Reductases
Penicillin; Cephalosporin; *Cephalosporium acremonium*; Blastospore; Actin; Cytochalasins; Cysteine; Glutathione (Crabbe, M.J.C. (56) 71)

Reductive pentose phosphate cycle
Reversed respiratory electron transport; Chemolithoautotrophy; Nitrogen fixation; *Bradyrhizobium japonicum* (Häger, K.-P. (56) 189)

Regulation
Anabaena doliolum; Urea-uptake; Cyanobacterium (Singh, S. (56) 281)

Restriction endonuclease
Acetobacter (Tagami, H. (56) 161)

Restriction map
Brucella phages; DNA (Segondy, M. (56) 177)

Reversed respiratory electron transport
Chemolithoautotrophy; Reductive pentose phosphate cycle; Nitrogen fixation; *Bradyrhizobium japonicum* (Häger, K.-P. (56) 189)

***Rhizobium leguminosarum* IARI 102**
Siderophore; Iron; 2,3-DHBA (Patel, H.N. (56) 131)

Rhodobacter capsulatus
Nif$^-$ mutants; Continuous culture; H_2 production (Willison, J.C. (56) 61)

Ribosomal mutant
Escherichia coli (Mikkola, R. (56) 265)

mRNA
Ammonia production; *Anabaena variabilis*; Glutamine synthetase; Mutant strain (Hien, N.T. (56) 337)

***R*-plasmid**
Corynebacterium diphtheria; Macrolide-lincosamide-streptogramin B resistance; Erythromycin resistance; Methylase gene; Spontaneous deletion (Serwold-Davis, T.M. (56) 7)

Ruthenium red
Plate assay; Fungal polygalacturonase (McKay, A.M. (56) 354)

Saccharomyces cerevisiae
Conditioning; Acquired osmotolerance; Acquired thermotolerance; Physiological overlap; *sn*-Glycerol 3-P dehydrogenase; Heat shock protein (Trollmo, C. (56) 321)

SDS PAGE
Sheep antibody; *Pasteurella haemolytica*; Antigen; Immunoblotting (Donachie, W. (56) 271)

Sequence
Maltogenic amylase; Cloning; *Bacillus subtilis* (Diderichsen, B. (56) 53)

Sewage
Disinfection; UV-irradiation; Chlorine; Bacteriophages; Tap water; *Bacteroides fragilis* (Tartera, C. (56) 313)

Sheep antibody
Pasteurella haemolytica; Antigen; SDS PAGE; Immunoblotting (Donachie, W. (56) 271)

Siderophore
Rhizobium leguminosarum IARI 102; Iron; 2,3-DHBA (Patel, H.N. (56) 131)

S-layer
Bacillus brevis; Cell wall (Gruber, K. (56) 113)

Spontaneous deletion
Corynebacterium diphtheria; Macrolide-lincosamide-streptogramin B resistance; Erythromycin resistance; Methylase gene; *R*-plasmid (Serwold-Davis, T.M. (56) 7)

Steroid-transforming enzyme
Monoclonal antibodies; *Streptomyces hydrogenans* (Kempf, B. (56) 237)

Streptococcus pyogenes
Transformation; Electroporation; Plasmid (Suvorov, A. (56) 95)

Streptomyces antibioticus
Phage adsorption; Adsorption; Endodeoxyribonuclease (De los Reyes-Gavilán, C.G. (56) 301)

Streptomyces hydrogenans
Monoclonal antibodies; Steroid-transforming enzyme (Kempf, B. (56) 237)

Strict aerobic growth
DNA gyrase; Anaerobiosis; Gene fusion; Nalidixic acid resistance; *gyrA* (Yamamoto, N. (56) 209)

Sulfolobus acidocaldarius
Heat shock response; *Thiobacillus ferrooxidans*; Thermotolerance; Protein synthesis; Ethanol (Jerez, C.A. (56) 289)

Tap water
Disinfection; UV-irradiation; Chlorine; Bacteriophages; Sewage; *Bacteroides fragilis* (Tartera, C. (56) 313)

Thermophile
Anaerobe; Carbohydrate utilisation (Patel, B.K.C. (56) 285)

Thermophiles
Yeast HSP70 gene; Evolutionary conservation/eubacteria/archaebacteria (Lambert, E. (56) 157)

Thermotolerance
Heat shock response; *Sulfolobus acidocaldarius*; *Thiobacillus ferrooxidans*; Protein synthesis; Ethanol (Jerez, C.A. (56) 289)

Thiobacillus ferrooxidans
Heat shock response; *Sulfolobus acidocaldarius*; Thermotolerance; Protein synthesis; Ethanol (Jerez, C.A. (56) 289)

Thymidine phosphorylase
Uridine phosphorylase; *Lactobacillus casei* (Avraham, Y. (56) 29)

Transformation
Bacillus subtilis; Protoplast; Plasmid (Rubinstein, C.P. (56) 67)

Clostridium acetobutylicum; Cloning vector; Electroporation (Oultram, J.D. (56) 83)

Streptococcus pyogenes; Electroporation; Plasmid (Suvorov, A. (56) 95)

Bordetella; Electroporation; Electric field pulses (Zealey, G. (56) 123)

Triangular bacterium
Cell plate; Cell division; Halobacterium; Cinematography (Hamamoto, T. (56) 221)

Trypanosoma cruzi
Glutamate dehydrogenase; NAD; NADP; Purification; Amino acid composition; N-terminal sequence (Cazzulo, J.J. (56) 215)

U937 cells
Legionella pneumophila; Conjugal DNA transfer; Counter-selectable plasmids; Allelic exchange (Cianciotto, N.P. (56) 203)

Urea-uptake
Anabaena doliolum; Cyanobacterium; Regulation (Singh, S. (56) 281)

Uridine phosphorylase
Thymidine phosphorylase; *Lactobacillus casei* (Avraham, Y. (56) 29)

UV-irradiation
Disinfection; Chlorine; Bacteriophages; Tap water; Sewage; *Bacteroides fragilis* (Tartera, C. (56) 313)

Vero toxin
Nicked and unnicked Shiga-like toxin; Cytotoxic activity (Kongmuang, U. (56) 105)

Vibrio alginolyticus
Adenosine triphosphatase; Cell membrane (Dmitriev, O.Y. (56) 79)

Vibrio vulnificus
Cytotoxin; Virulence (Massad, G. (56) 295)

Virulence
Cytotoxin; *Vibrio vulnificus* (Massad, G. (56) 295)

Virulence factor
Antral gastritis; Duodenal ulcer; Pathogenesis; Adhesin (Huang, J. (56) 109)

Yeast HSP70 gene
Thermophiles; Evolutionary conservation/eubacteria/archaebacteria (Lambert, E. (56) 157)

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Publication Schedule for 1989

	Sep-Dec 1988	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
FEMS Microbioloy Letters (L) (7 volumes)	**55-1** L46-1 **55-2/3** L46-2/3	**56-1** L47-1	**56-2 56-3** L47-2 L47-3	**57-1** L48-1	**57-2 57-3** L48-2 L48-3	**58-1** L49-1	**58-2 58-3** L49-2 L49-3	**59-1** L50-1	**59-2 59-3** L50-2 L50-3	**60-1** L51-1	**60-2 60-3** L51-2 L51-3	**61-1** L52-1	**61-2 61-3** L52-2 L52-3
FEMS Microbiology Ecology (E) (1 volume)		**62-1** E5-1		**62-2** E5-2		**62-3** E5-3		**62-4** E5-4		**62-5** E5-5		**62-6** E5-6	
FEMS Microbiology Reviews (R) (1 volume)		**63-1** R5-1			**63-2** R5-2			**63-3** R5-3			**63-4** R5-4		
FEMS Microbiology Immunology (I) (1 volume)		**64-1** I2-1		**64-2** I2-2		**64-3** I2-3		**64-4** I2-4		**64-5** I2-5		**64-6** I2-6	

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Methods for the identification and analysis of L-altrose in bacterial polysaccharides

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1. SUMMARY

Methods for the detection, quantitation, and configurational analysis of altrose in bacterial polysaccharides were developed and are described. Thin layer chromatography (TLC) is first used to qualitatively detect altrose and other monosaccharides in polysaccharide hydrolysates. Alditol acetates are then prepared from these hydrolysates and analyzed by either gas-liquid chromatography (GLC) or GLC-mass spectroscopy (GLC-MS), depending on the nature of the constituent sugars. These data allow the calculation of either the relative or absolute neutral sugar composition (using an internal standard) of a given polysaccharide. The absolute configuration of altrose and other constituent sugars in bacterial polysaccharides was easily determined by GLC analysis of acetylated diastereomeric glycosides prepared from chiral (−)-2-octanol and polysaccharide hydrolysates.

Extracellular polysaccharides (EPS) produced by *Butyrivibrio fibrisolvens* were analyzed by these techniques, and we report here that L-altrose was found in the EPS from five different strains of this organism.

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2. INTRODUCTION

Stack [1] recently reported that the extracellular polysaccharides (EPS) produced by *Buturivibrio fibrisolvens* strain CF3 contain L-altrose. This was the first reported natural occurrence of this unusual hexose, which is the C-5 epimer of D-galactose. The altrose content of the EPS from strain CF3 was easily determined by GLC analysis of the alditol acetates prepared from EPS hydrolysates. Altrose yielded two GLC peaks when analyzed by these methods, however, which were subsequently identified as 2,3,4-tri-0-acetyl-1,6-anhydroaltrose (Ac_3AnAlt) and altritol hexaacetate (Ac_6Alt). These results reflect the tendency of altrose to form 1,6-anhydroaltrose in acidic solutions. Potentially further complicating GLC analysis, the Ac_3AnAlt had the same retention time as fucitol pentaacetate (Ac_5Fuc), and the Ac_6Alt had the same retention time as mannitol hexaacetate

(Ac_6Man). However, since neither fucose nor mannose was found in the EPS made by strain CF3, these potential interferences were not a problem.

We have encountered in subsequent studies EPS from additional *B. fibrisolvens* strains that contained various combinations of altrose, fucose, mannose, and other sugars. It was necessary to develop general methods for the detection, quantitation, and configurational analyses of altrose in bacterial polysaccharides, which this report describes.

3. MATERIALS AND METHODS

3.1. Organisms, growth conditions and polysaccharide purification

Strains of *B. fibrisolvens* were all from the culture collection of R.B. Hespell, Northern Regional Research Center, USDA-ARS, Peoria, Illinois: strain H4a [2], strain ARD23c [3], strain PI-26 [4], strain R-28 (isolated by M.A. Rasmussen, University of Illinois, as a cellulolytic bovine rumen isolate), and strain C-14 (isolated by M.P. Bryant, University of Illinois, as a pectinolytic bovine rumen isolate). All cultures were grown anaerobically to stationary phase as previously described [1] on the chemically defined medium of Cotta and Hespell [5]. Extracellular polysaccharides (EPS) were purified from culture supernatants following lyophilization, dialysis, and phenol extraction as previously described [1].

3.2. Monosaccharide analyses

EPS hydrolysates were prepared by treating 5–10 mg of EPS in trifluoroacetic acid (TFA, 0.5 ml, 2 M, 121°C, 1 h), and were qualitatively analyzed for carbohydrate content by thin layer chromatography (TLC) on Whatman K-5 silica gel plates as previously described [1]. Quantitative neutral sugar content of EPS samples was determined by gas-liquid chromatography (GLC) of alditol acetate derivatives, using the modification of the Albersheim et al. [6] procedure previously described [1]. Peak areas, detected by flame ionization, were used to calculate neutral sugar content relative to galactitol hexaacetate. Inositol was routinely included as an internal standard.

3.3. Determination of the altrose content of EPS

The method used to determine the altrose content of a given EPS sample depended on the compositional features of the EPS noted by TLC analysis. For example, the altrose content of EPS devoid of both fucose and mannose was determined simply by adding the areas of the GLC-separated peaks corresponding to 2,3,4-tri-O-acetyl-1,6-anhydroaltrose (Ac_3AnAlt) and altritol hexaacetate (Ac_6Alt). The altrose content of EPS samples containing fucose (but devoid of mannose) was calculated solely from the area of the Ac_6Alt peak, as will be described more fully in section 4. Finally, the relative composition of EPS samples containing altrose, fucose, and mannose was determined using GLC/MS of prepared alditol acetate derivatives. Chemical ionization mass spectra were obtained with a Finnegan 4535/TSQ GLC/MS equipped with a 0.25 μm × 30 meter DB-225 fused silica capillary column (J and W Scientific, Rancho Cordova, CA). Injections of alditol acetates (1–3 μl) were split 15/1 and run isothermally at 210°C. Isobutane was used as the reagent gas (0.3 torr) and the source temperature was 100°C. The total yield of ions at m/e 289 (indicative of Ac_3AnAlt) and m/e 317 (indicative of Ac_5Fuc) was summed for individual scans taken across the Ac_3AnAlt/Ac_5Fuc peak. These data were used to deduce the individual mass contributions of Ac_3AnAlt and Ac_5Fuc to this peak. The amount of Ac_3AnAlt so determined allowed the calculation of the amount of Ac_6Alt (and Ac_6Man) in the Ac_6Alt/Ac_6Man peak, as will be described in more detail later.

3.4. Determination of the configuration of hexoses in EPS

The absolute configuration of neutral monosaccharides in EPS was determined by GLC analysis of their acetylated diastereomeric 2-octyl glycosides prepared from either (−)-2-octanol or (+)-2-octanol, using a modification of the procedure described by Leontein et al. [8]. EPS samples (4–8 mg) in 13 × 100 mm teflon-capped tubes were hydrolysed in trifluoroacetic acid (TFA, 500 μl, 2

M, 110 °C, 1 h) and then evaporated to dryness with a stream of N_2 at 60 °C. The residue was dissolved in TFA (10 μl, 13.5 M), (−)-2-octanol (400 μl) was added, and the resulting mixture was sonicated extensively in a bath sonicator at room temperature. The samples were then incubated at 100 °C for 12–16 h. Excess (−)-2-octanol/TFA was evaporated with a stream of N_2; complete removal of these reagents was accomplished by placing tubes on a vacuum pump for an additional 5–8 h. The resulting mixture of diastereomeric glycosides was acetylated with acetic anhydride (250 μl, 90 °C, 1h, containing 4 mg/ml dimethylaminopyridine, [9]), and then evaporated to dryness with a stream of N_2 at 60 °C. The products were dissolved in CH_2Cl_2 (500 μl), and 1–5 μl injections were made into a Hewlett Packard 5890A Gas Chromatograph (equipped with the same column mentioned above) run isothermally at 210 °C. Standards were prepared from D-glucose, L-glucose, D-galactose, L-galactose, D-mannose, L-mannose, D-fucose, L-fucose, D-altrose, L-rhamnose. Standards corresponding to L-altrose and D-rhamnose were prepared similarly from D-altrose and L-rhamnose by substitution of (+)-2-octanol for (−)-2-octanol, as these monosaccharides are not commercially available. All sugars and reagents used for these procedures were obtained from Sigma Chemical Co., St. Louis, MO. Partially purified altrose from the EPS of strain R-28 was also analyzed by the above methods, following paper chromatographic separation and elution as previously described [1].

4. RESULTS AND DISCUSSION

The determination of neutral sugar components in polysaccharides via GLC separation of alditol acetates is a well-established and often used analytical procedure. All of the commonly encountered alditol acetates are well-resolved by capillary GLC, and may be quantified either on a relative basis or by the use of an internal standard. Stack [1] recently reported however, that the EPS of *B. fibrisolvens* strain CF3 contained L-altrose, which when analyzed by these methods, yielded two distinct GLC peaks. One of these, Ac_3AnAlt, co-chromatographed with Ac_5Fuc and represented acetylated 1,6-anhydroaltrose, which forms from altrose in acidic solutions [1,7]. The other, Ac_6Alt, represented the expected hexitol hexaacetate of altrose and was found to co-chromatograph with Ac_6Man. Since the EPS of *B. fibrisolvens* strains CF3 was devoid of both fucose and mannose, the quantitative analysis of altrose in this EPS by conventional alditol acetate procedures presented no serious difficulties.

In screening other strains of *B. fibrisolvens* for altrose-containing EPS, we encountered several which yielded GLC peaks with retention times

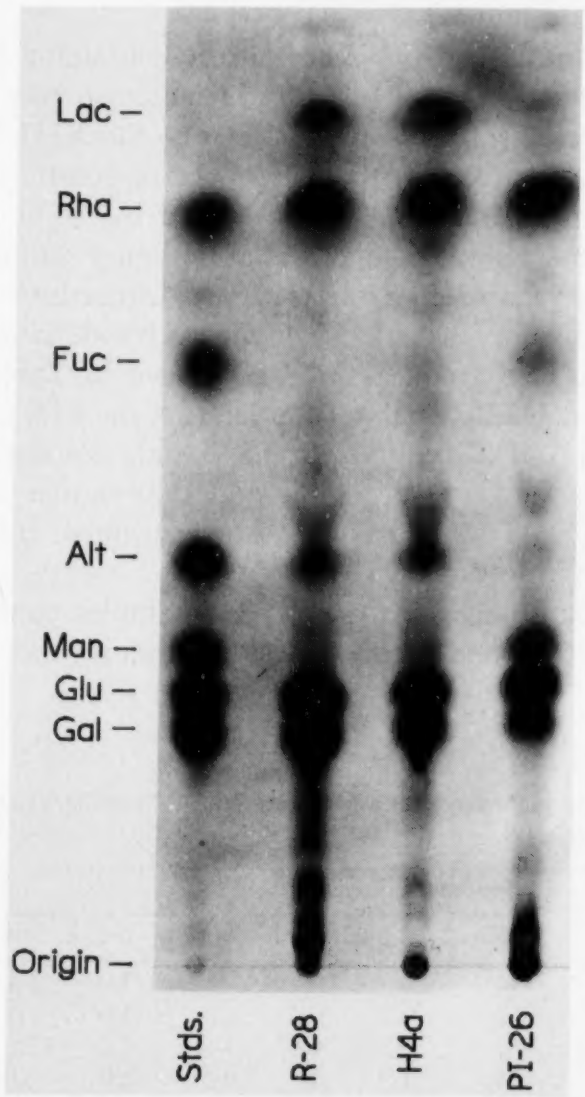

Fig. 1. TLC separation of monosaccharides obtained from acid hydrolysates of the EPS from *B. fibrisolvens* strains R-28, H4a, and PI-26. Gal, Glu, Man, Alt, Fuc, and Rha represent galactose, glucose, mannose, altrose, fucose, and rhamnose, respectively. 1,6-Anhydroaltrose is present at the leading edge of the Rha peak. Lac represents a lactone of an unidentified acidic sugar also present in these EPS (R.J. Stack, unpublished data).

corresponding to Ac_3AnAlt/Ac_5Fuc and Ac_6Alt/Ac_6Man. The EPS from these strains – ARD23c, C-14, H4a, PI-26, and R-28 – were then screened qualitatively for altrose, fucose, and mannose by TLC, as these sugars are well-resolved by this system. Representative results are shown in Fig. 1. The EPS of strain R-28 yields a spot coincident with authentic altrose, but none coincident with fucose or mannose. The EPS from strain H4a has spots coincident with both altrose and fucose, but not mannose. The EPS from PI-26 contains altrose, fucose, and mannose. Additional spots corresponding to rhamnose, galactose, and glucose were also observed in each of the three EPS hydrolysates examined.

Quantitative analysis of altrose-containing EPS samples devoid of fucose and mannose was determined as previously described by Stack [1]. The summed area of the peaks corresponding to Ac_3AnAlt and Ac_6Alt was divided by Ac_6Gal to give the relative monosaccharide composition of these types of EPS samples. This procedure was used to give the relative monosaccharide content of the EPS from strain R-28 shown in Table 1. Although TLC analysis showed that the EPS from strain ARD23c contained fucose (data not shown), the amount was negligible and the relative composition of this EPS was also determined by the above procedure.

Quantitative analysis of EPS samples containing altrose and fucose (but not mannose) was also readily determined from peak areas of GLC-separated alditol acetates. GLC analysis of the alditol acetates prepared from acid-treated standard altrose showed that the area of the peak corresponding to Ac_6Alt comprised 40% of the total area (Ac_3AnAlt and Ac_6Alt) identifiable as altrose. These results reflect the position of the acid-catalyzed equilibrium of altrose with 1,6-anhydroaltrose, and are in good agreement with data published in other studies [1,7]. Thus the total area corresponding to altrose in the EPS of strain H4a could be calculated simply by dividing the area of Ac_6Alt by 0.4. Back calculation of the area expected from the corresponding amount of Ac_3AnAlt, and substraction of this from the total area of the Ac_3AnAlt/Ac_5Fuc peak, gave the area due to Ac_5Fuc. An analogous procedure could be used to calculate the altrose and mannose content of EPS devoid of fucose, although no EPS of this nature was encountered in the present study.

Chemical ionization mass spectroscopy of GLC-separated alditol acetates was used to determine the relative composition of EPS samples containing altrose, fucose, and mannose. The base peak of Ac_3AnAlt was the protonated molecular ion at m/e 289 [1], while that of Ac_5Fuc was at m/e 317 $[M\text{-}59]^+$ (data not shown). These two ions were used diagnostically to identify these two compounds and calculate their relative mass contributions to the Ac_3AnAlt/Ac_5Fuc peak. The total number of ions at m/e 289 and m/e 317 (n_{289} and n_{317}) was determined by summing the values obtained from individual scans taken across the Ac_3AnAlt/Ac_5Fuc peak. Independent experiments with standards established that the yield of ions at m/e 317 (indicative of Ac_5Fuc) per unit of mass was 1.69 times greater than the yield of ions at m/e 289 (indicative of Ac_3AnAlt) under the described experimental conditions. Therefore, multiplication of the observed ratio of summed ions (n_{289}/n_{317}) by 1.69 yielded the actual mass ratio of Ac_3AnAlt/Ac_5Fuc in the peak. The amount of Ac_6Alt in the Ac_6Alt/Ac_6Man peak was calculated from the amount of Ac_3AnAlt so determined, and the relative proportion of fucose, altrose, and mannose obtained. This method was used to calculate the relative neutral sugar composition of the EPS produced by *B. fibrisolvens*

Table 1

Relative monosaccharide content of the EPS made by strains of *B. fibrisolvens*

Strain	Monosaccharide [a]					
	L-Rha [b]	L-Fuc	L-Alt	D-Man	D-Gal	D-Glu
R-28	0.2	–	0.5	–	1.0	1.1
H4a	0.2	0.1	0.6	–	1.0	1.3
ARD23c	0.2	tr [c]	0.9	–	1.0	2.6
C-14	1.4	1.3	1.5	0.2	1.0	6.0
PI-26	2.1	0.6	0.2	0.7	1.0	1.9

[a] Absolute configurations deduced from analysis of acetylated diastereomeric glycosides.

[b] L-Rha = L-rhamnose; L-Fuc = L-fucose; L-Atl = L-altrose; D-Man = D-mannose; D-Gal = D-galactose; and D-Glu = D-glucose.

[c] tr = trace, defined as less than 0.1.

strains C-14 and PI-26. The obtained results correlated with visual estimates of these sugars made on the basis of stained TLC plates (Fig. 1). The relative neutral sugar compositions of the EPS from the five strains of *B. fibrisolvens* determined by the above methods are presented in Table 1.

The absolute configuration of the neutral sugars in these EPS was determined by GLC analysis of the acetylated diastereomeric glycosides produced from EPS hydrolysates and chiral (−)-2-octanol. As discussed by Leontein et al. [8], a single isomer of a pure sugar yields four different glycosides (two pyranosides and two furanosides), following treatment with an optically-pure alcohol. After acetylation, these can usually be separated by GLC. The particular chromatographic pattern obtained from a given sugar is usually unique enough to allow the unambiguous assignment of its absolute configuration. Fig. 2A shows the GLC pattern of acetylated glyocosides obtained from acid-treated D-altrose and (−)-2-octanol. The expected four peaks are observed with retention times of 26.44, 27.87, 31.03, and 33.74 minutes. The additional peak seen at 8.08 min was identified as Ac_3AnAlt. This peak was observed only when the D-altrose was acid-treated prior to glycoside formation; otherwise there were corresponding proportional increases in the other four peaks (data not shown). However, since EPS samples were acid hydrolysed prior to glycoside formation, it was deemed appropriate to acid treat the standards used for comparative purposes.

Fig. 2B shows the GLC-separated acetylated glycosides prepared from (−)-2-octanol and acid hydrolysates of the EPS from R-28. Although some of the peaks in Fig. 2B match those seen in Fig. 2A, the large peak at 27.87 min is conspicuously absent. This suggested that the altrose in this EPS was not of the D-configuration. Since cochromatography of some of the acetylated glycosides obtained from other hexose monosaccharides was observed, it is of considerable import to note the absence as well as the presence of all diagnostic peaks before assigning absolute configurations.

Fig 2C shows a similar analysis of the acid-treated 'L-altrose standard', which was actually prepared from D-altrose and (+)-2-octanol

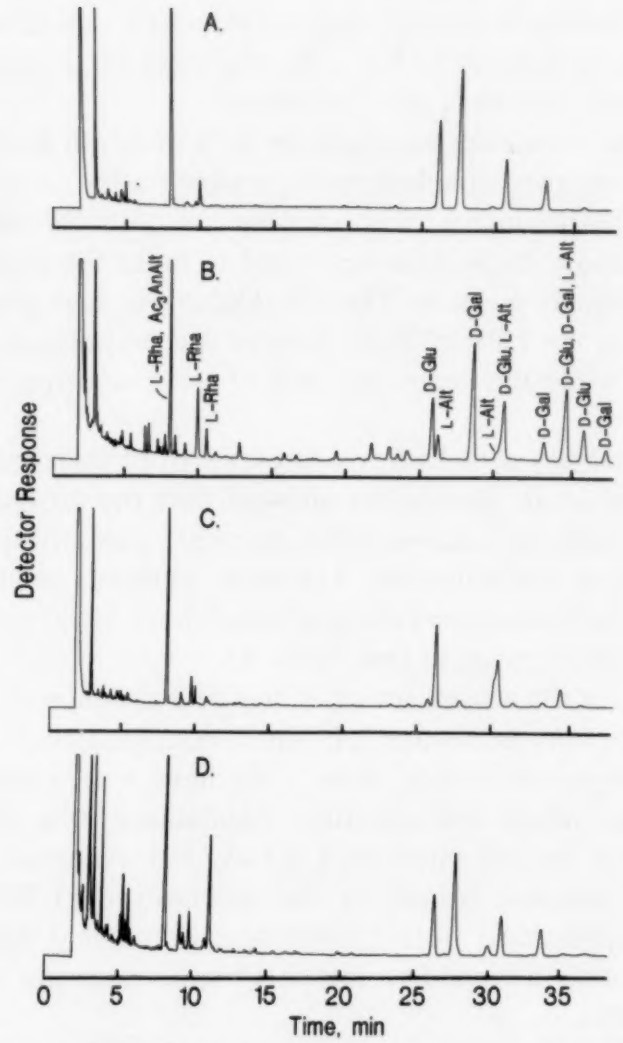

Fig. 2. Configurational analyses of hexoses by GLC of acetylated diastereomeric glycosides. (A) Pattern obtained from standard D-altrose and (−)-2-octanol. Note peaks at 8.08 (Ac_3AnAlt), 26.44, 27.87, 31.03, and 33.74 min. (B) Pattern obtained from acid hydrolysate of the EPS from strain R-28 and (−)-2-octanol. Note particularly the absence of the peak at 27.87 min. (C) Pattern obtained from standard D-altrose and (+)-2-octanol, representing the 'L-altrose standard'. (D) Pattern obtained from the altrose in the EPS of strain R-28 isolated by paper chromatography and (+)-2-octanol. Note the equivalence with Fig. 2A.

[8]. All of the observed peaks are seen to have counterparts in Fig. 2B. However, the possibility of cochromatography of acetylated glycosides from other sugars with diagnostic peaks precluded absolute confirmation that the altrose made by strain R-28 was of the L-configuration. Therefore, altrose from the EPS of strain R-28 was separated from other constituents by paper chromatography, eluted with water, and reanalyzed as above using (+)-2-octanol (Fig. 2D). Since the resulting pat-

tern matched exactly that of standard D-altrose and (−)-2-octanol (Fig. 2A), the isolated altrose was assigned the L-configuration.

The remaining sugars in the EPS of strain R-28 were assigned absolute configurations after analogous experiments with hexose monosaccharide standards. These data were used to make the peak assignment given in Fig. 2B. Galactose and glucose in the EPS of R-28 were of the D-configuration, while the rhamnose was of the L-configuration.

Analyses with EPS hydrolysates from the other strains of *B. fibrisolvens* showed that the altrose, rhamnose, and fucose (when present) were always of the L-configuration. Likewise, glucose, galactose, and mannose (when present) were always of the D-configuration (see Table 1).

Since the altrose found in the EPS produced by *B. fibrisolvens* always has the L-configuration, it seems probable that these cells have a C-5 epimerase which acts on either D-galactose or a derivative thereof (such as UDP-D-Gal). An analogous reaction results in the synthesis of UDP-L-iduronic acid from UDP-D-glucuronic acid. Experiments designed to test this hyphothesis are in progress.

These results conclusively show that L-altrose is a constituent of the EPS from five additional strains of *B. fibrisolvens*, and detail produces for the detection, quantification, and configurational analysis of this unusual hexose in sugar mixtures or EPS hydrolyzates. The danger of relying on alditol acetate procedures as a single and definitive method for neutral sugar analysis is readily apparent. Without corroborating mass spectral and TLC analyses, one cannot be sure of the identity of a GLC peak solely on the basis of its retention time. In the present case, these difficulties are illustrated by the cochromatography of Ac_5Fuc with Ac_3AnAlt and of Ac_6Man with Ac_6Alt. It is quite possible that other EPS in the literature which reportedly contain fucose and mannose may really contain altrose instead (or in addition).

The metabolic functions of altrose in particular, or of the EPS generally for *B. fibrisolvens* remain obscure. The EPS do not seem to serve as storage polysaccharides, as they remain relatively stable even after the death and lysis of bacterial cultures (R.J. Stack, unpublished data). Stack [1] has speculated that incorporation of L-altrose into these EPS may serve a protective function, preventing recognition and subsequent hydrolysis by the multitude of glycanase and glycosidase enzymes also present in the gastrointestinal tract. Assuming that the EPS do indeed serve a specific function(s), this might confer a selective advantage to these organisms.

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The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

REFERENCES

[1] Stack, R.J. (1987) FEMS Microbiol. Lett. 48, 83–87.
[2] Dehority, B.A. (1966) J. Bacteriol. 91, 1724–1729.
[3] Dehority, B.A. (1975) in Proc. First International Reindeer and Caribou Symp., Univ. of Alaska.
[4] Hazlewood, G.P., Theodorou, M.K., Hutchings, A., Jordan, D.J. and Galfre, G. (1986) J. Gen. Microbiol. 132, 43–52.
[5] Cotta, M.A. and Hespell, R.B. (1986) Appl. Environ. Microbiol. 52, 51–58.
[6] Albersheim, P., Nevins, D.J., English, P.D. and Karr, A. (1967) Carbohyd. Res. 5, 340–345.
[7] Angyal, S.J. and Dawes, K. (1968) Aust. J. Chem. 21, 2747–2760.
[8] Leontein, K., Lindberg, B. and Lönngren, J. (1978) Carbohydr. Res. 62, 569–583.
[9] Höfle, G., Steglich, W. and Vorbrüggen, H. (1978) Angewand. Chem. 17, 569–583.

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Identification of a methylase gene for erythromycin resistance within the sequence of a spontaneously deleting fragment of *Corynebacterium diphtheriae* plasmid pNG2

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1. SUMMARY

A 9.6 Mda *Corynebacterium diphtheriae* plasmid, pNG2, mediates inducible resistance to erythromycin and other macrolide-lincosamide-streptogramin B (MLS) antibiotics. Spontaneous deletion of a 1.2 Mda fragment of pNG2 leaves the derivative plasmid incapable of conferring MLS-resistance on its host. The pNG2 region containing this fragment was sequenced as were the regions flanking it. The fragment contained 1606 bp, was flanked by almost perfect 23 bp inverted repeats, and appeared to be excised precisely. It contained an open reading frame encoding a leader protein plus a protein which was similar in its amino acid sequence to the rRNA methylases produced by other erythromycin-resistant, Gram-positive bacteria. The *C. diphtheriae* gene was named *erm*Cd.

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2. INTRODUCTION

Plasmid-mediated erythromycin resistance (EmR) has been described for several Gram-positive and more recently for some Gram-negative bacteria. In EmR strains of *Corynebacterium diphtheriae*, resistance is mediated by a 9.6 Mda plasmid, pNG2 [1]. It was previously shown [2] that erythromycin resistance in *C. diphtheriae* strain S601 was of the dissociated, inducible macrolide-lincosamide-streptogramin B (MLS) type expressed by most clinical isolates of *Staphylococcus* and *Streptococcus* species. The MLS-resistant phenotype results from dimethylation of residue Ade 2058 of 23 rRNA of *Escherichia coli* (or its functional counterpart in other organisms) which lowers the affinity of rRNA for erythromycin [3–5].

The *C. diphtheriae* EmR plasmid pNG2 has several interesting features. Besides harboring a resistance gene it has the rare ability to replicate within both Gram-positive and Gram-negative bacteria [6]. In addition, the 9.6 Mda plasmid undergoes a frequent (10^{-3} per cell) deletion event. The spontaneous deletion of a 1.2 Mda fragment

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from pNG2 produces an 8.4 Mda derivative plasmid. Cells harboring the derivative instead of pNG2 are no longer resistant to erythromycin [1,7]. Restriction endonuclease mapping analyses place the 1.2 Mda deleting region within the 2.5 *Eco*RI fragment of pNG2 [7]. The *C. diphtheriae* EmR gene has been localized to that same *Eco*RI fragment by subcloning experiments [7]. These data suggests, at the least, that the deleting region overlaps the *C. diphtheriae* EmR gene.

In the present paper we report the DNA sequence for a portion of the pNG2 2.5 Mda *Eco*RI fragment which contains both a methylase gene and the spontaneously deleting region. The *C. diphtheriae* EmR gene product is compared to several methylase counterparts that confer erythromycin resistance on other Gram-positive organisms.

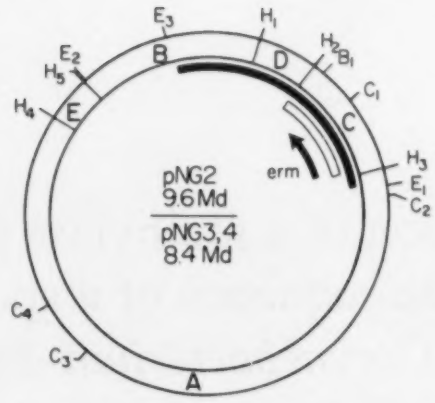

Fig. 1. *Hin*dIII map of pNG2 and pNG3/pNG4. *Hin*dIII sites are labeled H_1–H_5 and *Hin*dIII fragments are labeled A–E in order of their decreasing size. The remaining labeled restriction sites are *Bam*HI, B_1; *Cla*I, C_1–C_4; and *Eco*RI, E_1–E_3. The solid band marks the location of the 2.5 Mda *Eco*RI B fragment. The open band indicates the spontaneously deleting 1.2 Mda fragment associated with erythromycin resistance. The solid arrow represents the erythromycin resistance gene, as subsequently determined from sequencing, and is labeled 'erm.' pNG3 and pNG4 lack the 1.2 Mda fragment.

3. MATERIALS AND METHODS

3.1. Strains

C. diphtheriae strain S601(pNG2) and derivative strains S601-1(pNG3) and S601-16(pNG4) have been described previously [1]. *E. coli* strain JM103 [8] was the host strain used to propagate bacteriophage M13. Clones JM109(pNG2-5) and JM109(pNG3-2) have been described elsewhere [7] and were the respective sources of pNG2 and pNG3 DNA for subcloning into M13 phage vectors.

3.2. Media

C. diphtheriae strains were maintained on heart infusion (Difco Laboratories, Detroit, MI) plus 0.2% Tween 80 agar, with erythromycin added at 15 μg/ml when appropriate. *E. coli* strain JM103 was maintained on minimal A salts medium [9] and grown in 2 × TY consisting of 16 g bacto tryptine (Difco) 10 g yeast extract (Difco) and 5 g NaCl per liter, for infection with M13 phage. LB plates [9] with a 0.6% 2 × TY agar overlay were used for plaquing.

3.3. DNA procedures

C. diphtheriae plasmids and *E. coli* plasmids were isolated according to the procedures reported previously [7,10]. M13 phage subcloning, propagation and template preparation were all performed as described by Messing [11].

3.4. Subcloning

A set of deleted clones was made according to the manufacturer's suggestions, using the Cyclone System (IBI, New Haven, CT) which employs the T4 DNA polymerase enzyme. Three pNG2 subclones were deleted in this manner, two subclones containing the *Hin*dIII$_2$-*Hin*dIII$_3$ fragment of pNG2 (see Fig. 1) in M13mp19 in either orientation, and the third subclone, the pNG2 *Bam*HI-*Eco*RI$_1$ fragment in M13mp18. Subclone inserts were deleted in from one end starting at the unique *Eco*RI site in the M13 vector polylinker region. In this manner, a nested set of phage clones was created which allowed both strands of the entire *Hin*dIII$_2$-*Hin*dIII$_3$ stretch to be sequenced (see Fig. 2). Additional clones were obtained which spanned each of the *Hin*dIII sites so that pNG2 DNA outside of the *Hin*dIII C fragment could also be sequenced. The joining sites, formed in derivative plasmids pNG3 and pNG4 following deletion of the 1.2 Mda segment from pNG2, were also subcloned for sequencing by inserting the *Xba*I-*Eco*RI fragments of each plasmid (containing *Hin*dIII$_2$ and *Hin*dIII$_3$) into M13 phage vector M13mp18 (see Fig. 2).

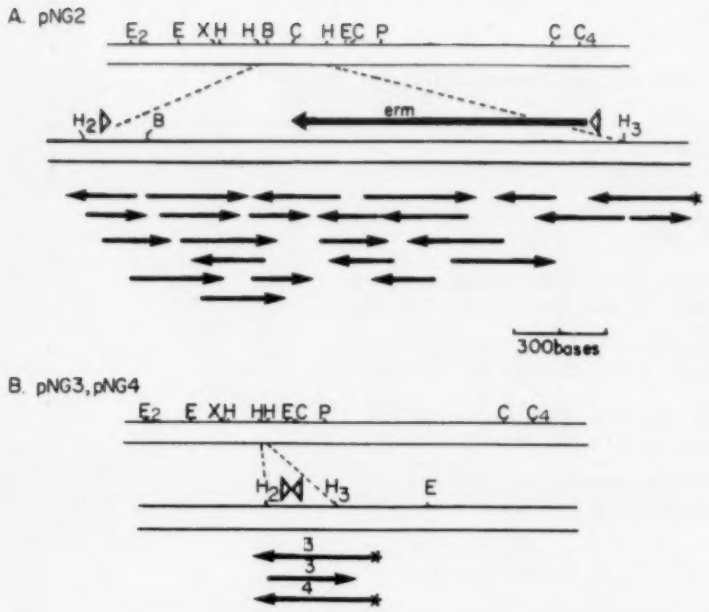

Fig. 2. Sequencing strategy. Both plasmids are drawn linearized. A. Sequencing strategy for the *Hin*dIII$_2$-*Hin*dIII$_3$ fragment which contains the *EmR* gene. B. Sequencing strategy for the region containing the joining sites in pNG3 and pNG4 resulting from the deletion of the 1.2 Mda fragment. The restriction sites are: E, *Eco*RI; X, *Xba*I; H, *Hin*dIII; B, *Bam*HI; C, *Cla*I; and P, *Pst*I. B. The sequences between H_2 and H_3 have been enlarged for clarity. The smaller, solid arrows denote the direction and length of the various sequencing reads. The pNG3 and pNG4 reads are labeled with a 3 or 4 respectively. Asterisks mark those reads obtained when the synthetic oligonucleotide primer was used. The locations of the EmR gene and the inverted repeats were subsequently determined by sequencing. The resistance gene is labeled 'erm,' and represented by the large solid arrow. Open arrowheads mark the location of the 23 basepair inverted repeats which flank the spontaneously deleting 1.2 Mda fragment of pNG2.

3.5. Sequencing

Dideoxy sequencing was performed using (α-^{32}P)dATP (Amersham, Arlington Heights, IL), specific activity of 800 Ci/mmol, according to the method of Sanger [12]. Klenow fragment of DNA polymerase I was purchased either from BRL, Gaithersburg, MD or Boehringer, Indianapolis, IN. Deoxyribonucleotides and dideoxyribonucleotides were purchased from Pharmacia P-L Biochemicals, Piscataway, NJ and the universal primer used was a 17-mer made by New England Biolabs, Beverly, MA. The oligonucleotide primer used to sequence three of the subclones was an 18-mer whose sequence (5′-AGGAAACAACAG-GTCTCA-3′) was chosen using data obtained for the region approximately 200 bases upstream from the *Hin*dIII$_3$ site. The oligomer was synthesized using the Biosearch DNA Synthesizer 8600, San Rafael, CA.

4. RESULTS AND DISCUSSION

The localization of the *C. diphtheriae* EmR gene was accomplished by sequencing the *Hin*dIII fragment of pNG2 which lies between *Hin*dIII$_2$ and *Hin*dIII$_3$ (See Fig. 1). This fragment was selected because in size, restriction site content, and location of its termini it closely approximated the region occupied by the 1.2 Mda spontaneously deleting fragment associated with erythromycin resistance. The sequencing strategy is diagrammed in Fig. 2A.

The sequence between the *Hin*dIII sites of pNG2 (Fig. 3) contained one long open reading frame (ORF) which had amino acid homology with known 23S rRNA methylase proteins produced by other erythromycin-resistant organisms (see below). The ORF spanned nucleotides 296 to 1150, and encoded a 284 amino acid protein with a calculated molecular mass of 31 690. The gene for this protein has been designated *erm*Cd. The *erm*Cd structural gene is preceded by a strong Shine-Dalgarno sequence [13] at nucleotides 283–288. The *erm*Cd message is most likely transcribed starting at adenine residue 150, 8 bases downstream from a nearly perfect polymerase binding site [14], composed of the −10 and −35 regions underlined in the figure. No other likely promoter sequences could be found for 600 bases upstream from the *erm*Cd ORF. A possible stem and loop structure in the DNA which followed the *erm*Cd ORF and may act as a transcriptional terminator is underlined in Fig. 3 between nucleotides 1148 and 1218.

The *erm*Cd message appears to encode a 21 amino acid leader peptide at its 5′ end from nucleotide 150 to 215. The presence of a leader peptide on the message coded for by *emr*Cd follows the pattern seen with other methylase genes. However, assuming transcription and translation begin at the sites we have proposed, *erm*Cd differs from previously studied *erm* genes in lacking a Shine-Dalgarno (S-D) sequence and lacking the

pNG2

```
HindIII3 .                                                                    .
AAGCTTGGGCTACCGCACGCCAGCTGAAGTGGAAACCGAATTTTGGGACCAGCACCCGCCGCAAGCAATAATAGAAATCAAGGCAAACGCCTAGGAACAA      100
                                                  message start                                  23 bp
          .   -35   .         .        -10.       .         .         .         .         .
AACCCGGGGCACTTCACACCCGCGAATTTAAATGGATATAATTAGACCC ATG TTG ATT TCA GGT ACC GCT TTC TTG CGG TTG CGC ACC      188
 inv. repeat   *                                   Met Leu Ile Ser Gly Thr Ala Phe Leu Arg Leu Arg Thr
 .         .         .         .         .         .         .         .         .         .
AAC CGT AAG GCG TTT CCT ACC CCG TAG GTTAAAAACCGCTCCGGTCCTTTAGGCCGGGCGGCTATTTGTCATGCCCGGCTCCCTTTCAAAT      279
Asn Arg Lys Ala Phe Pro Thr Pro  x
 .         .         .         .         .         .         .         .         .         .
CCAAGGAGCACACCTG ATG TCT GCA TAC GGA CAG GGC CGT CAC GAG CAT GGC CAA AAT TTT CTC ACC AAC CAC AAG ATC      358
   S-D           Met Ser Ala Tyr Gly Gln Gly Arg His Glu His Gly Gln Asn Phe Leu Thr Asn His Lys Ile
 .         .         .         .         .         .         .         .         .         .
ATC AAC TCC ATC ATC GAC CTT GTG AAA CAA ACC TCC GGC CCC ATC ATT GAG ATC GGA CCA GGA AGC GGT GCC CTC      433
Ile Asn Ser Ile Ile Asp Leu Val Lys Gln Thr Ser Gly Pro Ile Ile Glu Ile Gly Pro Gly Ser Gly Ala Leu
 .         .         .         .         .         .         .         .         .         .
ACT CAC CCG ATG GCC CAC TTG GGG AGG GCG ATA ACG GCA GTT GAA GTG GAC GCA AAA CTA GCT GCC AAA ATC ACA      508
Thr His Pro Met Ala His Leu Gly Arg Ala Ile Thr Ala Val Glu Val Asp Ala Lys Leu Ala Ala Lys Ile Thr
 .         .         .         .         .         .         .         .         .         .
CAA GAA ACC TCC TCG GCG GCG GTC GAA GTG GTC CAT GAT GAT TTC CTT AAC TTC CGG TTA CCC GCC ACT CCC TGC      583
Gln Glu Thr Ser Ser Ala Ala Val Glu Val Val His Asp Asp Phe Leu Asn Phe Arg Leu Pro Ala Thr Pro Cys
 .         .         .         .         .         .         .         .         .         .
GTC ATT GTG GGA AAC ATT CCC TTT CAC CTC ACC ACT GCC ATT CTT CGA AAG TTG CTG CAT GCG CCA GCA TGG ACT      658
Val Ile Val Gly Asn Ile Pro Phe His Leu Thr Thr Ala Ile Leu Arg Lys Leu Leu His Ala Pro Ala Trp Thr
 .         .         .         .         .         .         .         .         .         .
GAC GCT GTA CTC CTC ATG CAG TGG GAA GTC GCT CGC CGC CGG GCC GGG GTA GGC GCA ACG ACG ATG ATG ACG GCT      733
Asp Ala Val Leu Leu Met Gln Trp Glu Val Ala Arg Arg Arg Ala Gly Val Gly Ala Thr Thr Met Met Thr Ala
 .         .         .         .         .         .         .         .         .         .
CAG TGG TCC CCA TGG TTC ACA TTT CAC CTG GGT TCT CGG GTA CCA AGG TCT GCT TTC CGG CCA CAG CCA AAC GTT      808
Gln Trp Ser Pro Trp Phe Thr Phe His Leu Gly Ser Arg Val Pro Arg Ser Ala Phe Arg Pro Gln Pro Asn Val
 .         .         .         .         .         .         .         .         .         .
GAC GGG GGG ATC TTA GTG ATC CGC CGG GTG GGT GAC CCG AAG ATT CCG ATA GAG CAG CGC AAA GCC TTT CAG GCG      883
Asp Gly Gly Ile Leu Val Ile Arg Arg Val Gly Asp Pro Lys Ile Pro Ile Glu Gln Arg Lys Ala Phe Gln Ala
 .         .         .         .         .         .         .         .         .         .
ATG GTG CAC ACC GTT TTC ACT GCC CGG GGA CGC GGG ATA GGG GAA ATT CTC CGA AGG CAG GGT TGT TTT CAT CAC      958
Met Val His Thr Val Phe Thr Ala Arg Gly Arg Gly Ile Gly Glu Ile Leu Arg Arg Gln Gly Cys Phe His His
 .         .         .         .         .         .         .         .         .         .
GTT CAG AAA CAT AAT CAT GGT TGC GCT CGC GAG GAA TCG ACC CCG CGA CCC TAC CTC CCA GAT TGC TAC ACC AAC     1033
Val Gln Lys His Asn His Gly Cys Ala Arg Glu Glu Ser Thr Pro Arg Pro Tyr Leu Pro Asp Cys Tyr Thr Asn
 .         .         .         .         .         .         .         .         .         .
GAC TGG ATC GAT CTC TTC CAG GTG ACT GGT TCC TCT CTA CCT CAC CAT CGA CCC ATT TCA CCA TCG GGA AGT AGT     1108
Asp Trp Ile Asp Leu Phe Gln Val Thr Gly Ser Ser Leu Pro His His Arg Pro Ile Ser Pro Ser Gly Ser Ser
 .         .         .         .         .         .         .         .         .         .
CAA CGA CCT CCT CAA CGG AAA AAC CGA AGC CGG CGG CGT TAA TCCCCACCAAAACCGAAATCCACCAGTAGTACTAAAAGGGCTCT     1194
Gln Arg Pro Pro Gln Arg Lys Asn Arg Ser Arg Arg Arg  x                                          <-------
 .         .         .         .         .         .         .         .         .         .
TCCGGTTTTAGAGTGCATTGATTAGTTCGCCGGGGGTTCGGGCGTGGCACAACATATTGGGGTCCTTGGCTTTGTAGGAAAAAGGTATCTAATCCATCCG     1294
 ---------->
 .         .         .         .         .         .         .         .         .         .
TACAAAACTAAGGACCTACTGTGCAGCCCTAACGGAAATGTCATCGTCGATACCATCTGCCGCACCGCAAAACTAGGAACTACTATCACCGGTGCCACAG     1394
 .         .         .         .         .         .         .         .         .         .
AAAACGGTGACGTCACTGTTATTGAAGCCGAACCCGTCGAGCCGATCAATGAATGCCCTACCTGCGGGCAGCCTGGAGTATTCCGCGACCACGTCATCCG     1494
 .         .         .         .         .         .         .         .         .         .
CAGCCTGGTCGATCTGCCCATCGTCGGCCACCCAACAACTTCATGTGCGCCTTCCACGCTACCGGTGCACCAACAAGCGTTGCTTGCAGAAGATCTTCCG     1594
 .         .         .         .         .         .         .  BamHI  .         .
TGCTGGCCTTGCCTGCGCGCCCGACAATTCAAAAGACCACGGACCAGGTGACCCACTGGATCCTGCAACGACTCTCCTAAATCGGATGAGCGTTGCCGCT     1694
 .         .         .         .         .         .         .         .         .         .
GCACCCAAGTCATTAGGCCTGGGGTGGGATATGAAGTGTCCCAGGTTTTGTTCCGTTTGATGAAACCCGTTTTTGAAACAACGTACGGAGCACAACGCAC     1794
                          *     23 bp inv. repeat
    .  HindIII2
GCCGATGCCAAAGCTT                                                                                      1810
```

pNG3, pNG4

```
HindIII3 .                                                                    .
AAGCTTGGGCTACCGCACGCCAGCTGAAGTGGAAACCGAATTTTGGGACCAGCACCCGCCGCAAGCAATAATAGAAATCAAGGCAAACGCCTAGGAACAA      100
                                                                                          23 bp
                                                                                                  HindIII2
AACCCGGGGCACTTCAATATGAAGTGTCCCAGGTTTTGTTCCGTTTGATGAAACCCGTTTTTGAAACAACGTACGGAGCACAACGCACGCCGATGCCAAAGCTT 204
 inv. repeat          23 bp inv. repeat
```

Fig. 3. DNA sequences of the pNG2 erythromycin resistance associated region, *erm* gene and joining sites of pNG3 and pNG4. The negative DNA strand between *Hin*dIII$_2$ and *Hin*dIII$_3$ is presented for both pNG2 and pNG3/4. The *Hin*dIII$_{2/3}$ restriction sites are underlined and labeled, as are the 3 basepair inverted repeats. The amino acids encoded within the two open reading frames are given. The -35 and -10 regions of the promoter, and the Shine-Dalgarno sequence for the *erm* structural gene are labeled and the proposed transcriptional start site is also marked. '*x*' indicates the ends of the leader peptide and methylase protein and a stop or missense codon. Thin, long arrows underline a possible transcriptional termination signal. Asterisks mark the first and last bases of the pNG2 deleting fragment.

```
ermC                                                                  M N E K N I K H S Q N F I T S
ermAM                                                                 M K K N I K Y S Q N F L T N
ermD                                          M K K K N H K Y R G K K L N R G E S P N F S G Q H L M H N
ermE                                                  M L G R T E R D R N R R Q F G Q N F L R D
ermA      M A G P Q D R P R G R G P S S G R P Q R P V G G R S Q R D R D R R V L G Q N F L R D
ermCd                                                 M S A Y G Q G R H E H G Q N F L T N

ermC      K H N I D K I M T N I R L N E H D N I F E I G S G K G H F T L E L V K R C N F V T A
ermAM     E K V L N Q I I K Q L N L K E T D T V Y E I G T G K G H L T T K L A K I S K Q V T S
ermD      K K L I E E I V D R A N I S I D D T V L E L G A G K G A L T T V L S Q K A G K V L A
ermE      R K T I A R I A E T A E L R P D L P V L E A G P G E G L L T R E L A D R A R Q V T S
ermA      P A T I R R I A D A A D V D P D G L V V E A G P G E G L L T R E L A R R A G R V R T
ermCd     H K I I N S I I D - L V K Q T S G P I I E I G P G S G A L T H P M A H L G R A I T A

ermC      I E I D H K L C - K T T E N K L V D H D N F Q V L N K D I L Q F K F P K N Q S Y K I
ermAM     I E L D S H L F - N L S S E K L K L N I R V T L I H Q D I L Q F Q F P N K Q R Y K I
ermD      V E N D S K F V - D I L T R K T A Q H S N T K I I H Q D I M K I H L P K - E K F V V
ermE      Y E I D P R L A - K S L R E K L S G H P N I E V V N A D F L T A E F P P - E P F A F
ermA      Y E L D Q R L A - R R L S T D L A Q E T S I E V V H A D F L T A P H P E - E P F Q F
ermCd     V E V D A K L A A K I T Q E - T S S A A - V E V V H D D F L N T R L P A - T P C V I

ermC      Y G N I P Y N I S T D I I R K I V F D S - I A N E I Y L I V E Y - G F A K R L L N -
ermAM     V G S I P Y H L S T Q I I K K V V F E S - H A S D I Y L I V E E - G F Y K R T L D -
ermD      V S N I P Y A I T T P I M K M L L A A P A S G F Q K G I I V M E K G A A K R F T S K
ermE      V G A I P Y G I T S A I V D W C L E A P - T I E T A T M - V T Q L E F A R K R T G D
ermA      V G A I P Y G I T S A I V D W C L T A P - T L T S A T L - V T Q Q E F A R K R T G D
ermCd     V G N I P F H L T T A I L R K L I H A P A - W T D A V L - L M Q W E V A R R R A G -

ermC      - T K R S - L A L L L M A E V D I S I L S M V P R E Y F H P K P K V N S S L I - R L
ermAM     - I H R S - L G L L L H T Q V S I Q Q L L K L P A E C F H P K P K V N S V L I - K L
ermD      F I K N S - Y V L A W R M W F D I G I V R E I S K E H F S K P P K V D S A - - M V R
ermE      Y G R W S R L T V M T W P L F E W E F V E - - - - - - - - - K V D S A - I M R L
ermA      Y G R W T A L T V T T W P T F E W Q Y V A K V D R T L F T P V P R V H S A - I M R L
ermCd     V G A T T M M T A Q W S P W F T F H L G S R V P R S A F R P Q P N V D G G - I L V I

ermC      S R K K S R I S H K D K Q K Y N Y F V M K W V N K E Y K K I F T K N Q F N N S L K H
ermAM     T R H T T D V P D K Y W K L Y T Y F V S K W W N R E Y R Q L F T K N Q F H Q A M K H
ermD      I T R K K D A P - L S H K H Y - I A F R G L A E Y A L K E P N I P L C V A L R - - G
ermE      - R R R A E - P L L E - G A A L E R Y E S M V E L C F T G V G G N I Q A S L L R K Y
ermA      - R R R P Q - P L L R D A A A R S R F A D M V E I G F V G K G G S L Y R S L T R E W
ermCd     - R R V G D - P - K I P I E Q R K A F Q A M V H T V F T A R G R G I G E I L R R Q G

ermC      A G I D D - L N N I S F E Q F L H A G I D - D L N N - - - I S F E Q F L S L F N S Y
ermAM     A K - - V - - N N L S T V T Y E H A - - - - K V N N L S T V T Y E Q V L S I F N S Y
ermD      I F T P R Q M K H L R K S L K I N L R K S L K I N N E K T V G T L T E - - - - - - -
ermE      P R R R V - - E A A F D H A G V G - R K Y P R R R V E A A F D H A G V G G G A V V A
ermA      P R S K V - - D S A F A R A D V H H D E I - - - - - - - - - - - - - - - - - - V A
ermCd     C F H H V Q - K H N H G C A - - - - R E E S T P R P Y L P D C Y - T N - - - - - - -

ermC      - - - - - - - - - K L F N K . (244)
ermAM     - - - - - - - - - L L F N G R K . (245)
ermD      - - T E N Q W A V I F N T M T Q Y V M H H H K W P - - - - - - R A N K R K P G E I . (288)
ermE      Y V R P E Q W L R L F E R L D Q K N E P R G G Q P Q R G R R T G G R D H G D R R T G
ermA      F V H P D Q W I T L F Q L L D G S R G G A A R G P G D Q R G R R G R P G G G P R P D
ermCd     - - - - D - W I D L F Q V T G S S L P H H R P I S P S G S S Q R P P Q R K N R S R R R .(284)

ermE      G Q D R G D R R T G G R D H R D R Q A S G H G D R R S S G R N R D D G R T G E R E Q
ermA      G R A G G G P R R D A G G R R T G D G R G G R P R P P R G G Q A . (340)

ermE      G D Q G G R R G P S G G G R T G G R P G R R G G P G Q R . (345)
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Fig. 4. Comparison of amino acid sequence of the *erm*Cd gene product to five other methylase proteins. The strains encoding each of the methylases are *erm*C, *Staphylococcus aureus; erm*AM, *Streptococcus sanguis; erm*D, *Bacillus licheniformis; erm*A, *Arthrobacter* sp.; *erm*E, *Streptomyces erythreus;* and *erm*Cd, *Corynebacterium diphtheriae*. The number of amino acids in the protein is given in parentheses at the end of each sequence. The sequences have been aligned subjectively to give the best homology. Amino acids aligned with and identical to those of *erm*Cd are boxed.

possibility of an energetically stable stem-and-loop structure at the start of the leader peptide. At least two other examples have been reported in which translation takes place without a S-D signal [15,16]. In those cases, lack of a S-D signal results in inefficient translation. Though there are some possibilities of secondary structure formation in the 5′ leader region of the inducible *C. diphtheriae erm*Cd gene, these configurations are not as energetically stable as those reported for *S. aureus erm*C in which stem-and-loop structures in that region have been implicated in induction of the resistance phenotype [17,18].

The absence of a S-D signal for the *erm*Cd

leader peptide and less than optimal stability of possible stem-and-loop structures indicate that translation of the *erm*Cd mRNA will not follow the precise model developed for the *S. aureus erm*C gene in which both of these elements play a crucial role [17,18]. Nevertheless, induction of *erm*Cd expression is observed in the presence of erythromycin [2]. While an unstable stem-and-loop structure might permit constitutive synthesis of methylase, the absence of an S-D signal would buffer that tendency by reducing the efficiency of translation. Given these circumstances one would predict synthesis of a basal level of methylase in strains carrying pNG2, a level that might be elevated by further destabilization of the stem-and-loop structure on the addition of erythromycin. In fact, *C. diphtheriae* carrying pNG2 exhibit a fairly high basal level of resistance to erythromycin 32–256 μg without induction and increased resistance to the antimicrobic agent 500 μg/ml after induction [2].

The MLS-resistance genes (*erm* genes) in several Gram-positive and Gram-negative strains have been cloned and sequenced [19–26], and each encodes a methylase that modifies 23S ribosomal RNA. The deduced amino acid sequence of some of those methylase genes were recently compared, and their similarity established [25–27]. The amino acid sequence deduced from *erm*Cd was aligned with those deduced for *erm* gene products of five other Gram-positive organisms (Fig. 4). The alignment against which it was compared follows that presented by Uchiyama and Weisblum for some of the *erm* genes [26,27], and the sequences were spaced to give the closest possible match. The leader peptide sequences are not included since they seem to be quite variable, even between closely related *erm* genes. *erm*Cd shared the most amino acids with *Arthrobacter*, then *Streptomyces* and *Bacillus*, and the least with *Streptococcus* and *Staphylococcus*, although all were in the range of 18–30% amino acid identity with the *C. diphtheriae* protein. In each case the G + C content of the *erm* genes was similar to that established for their respective hosts suggesting that they have resided in these hosts for some time. We concluded from the significant amount of amino acid homology to other methylase proteins, the conserved spacing with which that homology is observed, and the inducibility of the *erm*Cd resistance phenotype that *erm*Cd coded for a ribosomal RNA methylase.

The sites at which spontaneous deletion of the *erm*C-carrying fragment occurred in pNG2 were determined next. Approximately 300 bp centering around the region of rejoining in the derivative plasmid pNG3 were sequenced according to the strategy shown in Fig. 2B. The sequence is given in the lower portion of Fig. 3. The *Hin*dIII$_2$ and *Hin*dIII$_3$ sites located on pNG2 were also present in pNG3. The sequences of plasmids pNG2 and pNG3 between these *Hin*dIII sites were identical up to and including base No. 116. Identity between their sequences resumed at pNG2 base No. 1723 and pNG3 base No. 117. The spontaneously deleting fragment contained exactly 1606 bases.

When the region at and around the rejoining site in pNG3 was examined, a pair of 23 base pair inverted repeats was detected which differed from each other at only two sites. Their homology and their proximity to the site of deletion suggests they are accessories in the excision event. The upstream repeat directly abutted the deleting sequence, while the downstream repeat was separated from it by three basepairs (Fig. 3). To find out whether this spacing was the result of a random or specific excision event, the same region was subcloned and sequenced from pNG4, a second independently generated EmS derivative of pNG2. The sequence for pNG4 was identical with that for pNG3, i.e. with the 'ATA' left between the two inverted repeats. This identity suggests a precise enzyme-mediated mechanism of excision. The association of inverted repeats with the excision event seems to rule out the possibility of a site-specific excision since that requires direct repeats.

The biological significance of the excision of the *erm*Cd-containing fragment from pNG2 cannot be evaluated as yet. Whether transfer of the fragment from one replicon to another occurs is uncertain. Independent transfer seems unlikely, since an ORF capable of coding for an excisionase has not been detected within the fragment. Such an enzyme might be present elsewhere on the plasmid or on the host's chromosome. We have found that the DNA of a plasmidless EmR

coryneform hybridized with a probe derived exclusively from the excised fragment (unpublished observations). This and the presence of inverted repeats at the termini of the fragment supports the idea of transposability.

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REFERENCES

[1] Schiller, J., Groman, N. and Coyle, M. (1980) Antimicrob. Agents Chemother. 18, 814–821.
[2] Coyle, M.B., Minshew, B.H., Bland, J.A. and Hsu, P.C. (1979) Antimicrob. Agents Chemother. 16, 525–527.
[3] Graham, M.Y. and Weisblum, B. (1979) J. Bacteriol. 137, 1464–1467.
[4] Skinner, R., Cundliffe, E. and Schmidt, F.J. (1983) J. Biol. Chem. 258, 12702–12706.
[5] Weisblum, B. (1975) in Microbiology-1974 (Schlessinger, ed.), pp. 199–206. American Society for Microbiology, Washington D.C.
[6] Serwold-Davis, T.M., Groman, N. and Rabin, M. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 4964–4968.
[7] Serwold-Davis, T.M. and Groman, N. (1986) Antimicrob. Agents Chemother. 30, 69–72.
[8] Kushner, S.R. (1978) in Genetic Engineering (Boyer, H.W. and Nicosia, S., eds.), pp. 17–23. Elsevier/North Holland Biomed. Press, Amsterdam, The Netherlands.
[9] Miller, J.H. (Ed.) (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
[10] Schiller, J., Strom, M., Groman, N. and Coyle, M. (1983) Antimicrob. Agents Chemother. 24, 892–901.
[11] Messing, J. (1983) Methods Enzymol. 101, 20–79.
[12] Sanger, F., Nicklen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467.
[13] Shine, J. and Dalgarno, L. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1342–1346.
[14] Rosenburg, M. and Court, D. (1979) Ann. Rev. Genet. 13, 319–353.
[15] Pirrotta, V. (1979) Nucleic Acids Res. 6, 1495–1508.
[16] Ptashne, M., Backman, K., Humayun, M.Z., Jeffrey, A., Maurer, R., Meyer, B. and Sauer, R.T. (1976) Science 194, 156–161.
[17] Horinouchi, S., and Weisblum, B. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7079–7083.
[18] Gryczan, T.J., Israeli-Reches, M. and Dubnau, D. (1980) Nucleic Acids Res. 8, 6081–6097.
[19] Mayford, M. and Weisblum, B. (1985) J. Mol. Biol. 185, 769–780.
[20] Gryczan, T., Israeli-Reches, M., Del Bue, M. and Dubnau, D. (1984) Mol. Gen. Genet. 194, 349–356.
[21] Horinouchi, S. Byeon, W.H. and Weisblum, B. (1983) J. Bacteriol. 154, 1252–1262.
[22] Monod, M., Mohan, S. and Dubnau, D. (1987) J. Bacteriol. 169, 340–350.
[23] Murphey, E. (1985) J. Bacteriol. 162, 633–640.
[24] Rasmussen, J.L., Odelson, D.A. and Macrina, F.L. (1986) J. Bacteriol. 168, 523–533.
[25] Roberts, A.N., Hudson, G.S. and Brenner, S. (1985) Gene 35, 259–270.
[26] Uchiyama, H. and Weisblum, B. (1985) Gene 38, 103–110.
[27] Uchiyama, H. and Weisblum, B. (1985) in Microbiology-1985 (Levie, L., Ed.), pp. 375–378. American Society for Microbiology, Washington, D.C.

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Spherical and rod shaped protoplasts from β-lactam antibiotic treated cultures of *Bacillus subtilis*

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1. SUMMARY

Addition of β-lactams to exponentially growing cultures of an autolytically deficient *Bacillus subtilis metc*3 *lyt*-2 strain FJ6 caused increase in optical density to stop after 1 h when it had about doubled, and thereafter to remain constant for at least 6 h. The number of protoplasts to be derived per unit dry weight of bacteria started to fall when the antibiotic was added and after 1 h had reached 50% of the initial value. Also during the first hour but after a lag of 20–30 min an increasing number of membrane bound rod shaped protoplasts were seen among the normal spherical ones. These swelled to spheres and ultimately burst if the concentration of sucrose in the suspending fluid was gradually reduced. These results would be best explained by rapid inhibition of initiation of cell division followed by membrane damage to an increasing proportion of cells.

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2. INTRODUCTION

The primary sites of action of β-lactam antibiotics are the integral membrane 'penicillin binding proteins' some or all of which are involved in the later stages of peptidoglycan synthesis [1]. Inhibition of transpeptidase action is the best defined result which in itself is likely to halt bacterial growth. Autolysins hydrolysing peptidoglycan present in almost all bacteria then play a large part in converting this bacteriostatic effect to a bactericidal one [2,3]. Nevertheless, some bacteria not possessing detectable autolytic activity (e.g. Group A *Streptococcus pyogenes* [4,5] or strains deprived of autolysis either genetically or phenotypically are still killed by the antibiotics at an appreciable rate [6,7] by so-called non-lytic death [8]. Very soon after the application of the antibiotics to rapidly growing cultures of such organisms changes suggesting membrane damage occur [6,9] and even RNA synthesis may be inhibited and ribonuclease activated [5]. In the present paper β-lactam antibiotics are shown to inhibit secondary cell-division sites in a *Bacillus subtilis lyt* strain followed shortly

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afterwards by such severe membrane damage to a proportion of the bacterial population that spherical protoplasts can no longer be formed.

3. MATERIALS AND METHODS

3.1. Microorganisms, media, and culture technique

B. subtilis met lyt-2 strain FJ6 [10] and *B. subtilis trp*C strain 168 were kept as spore suspensions at −20°C. The medium was acid hydrolysed casein, CHSC [11] supplemented by 20 μg/ml tryptophan for strain 168 or 20 μg/ml methionine for strain FJ6. Cultures were always grown in conical or side-arm flasks of five times their volume at 35°C in an orbitally shaking incubator. Inoculation was by placing 1 μl of spore suspension per ml of culture medium into a flask in the incubator and 8–9 h before the start of the experiment the required volume of sterile medium was added aseptically by a time-activated pump. Exponentially growing cultures were thus available at the start of the day.

3.2. Protoplast formation

Samples of cultures were mixed with equal volumes of Na^+/K^+ phosphate buffer (pH 6.6, 0.05 M) containing 1.0 M sucrose, 100 μg/ml lysozyme and 20 μg/ml DNase and incubated at 35°C for 1 h. Only phase dark spherical protoplasts remained when control cultures were so treated.

3.3. Protoplast counting

Visual counts were made using phase-contrast microscopy, with a magnification of 400 and an 'Improved Neubauer' counting chamber (Gallenkamp Ltd.). The number of protoplasts in 10 randomly selected 4 nl volumes was counted, each volume containing about 100 protoplasts. An electronic Coulter ZF particle counter was also used with a 30 μm orifice, an aperture current of 32 mA, amplitude of 0.177 and a threshold setting of 16. The protoplasts were first fixed by mixing suspensions of them with equal volumes of Na^+/K^+ phosphate buffer (pH 6.6, 0.05 M) containing 0.5 M sucrose and 10% (v/v) formalin. The suspensions stood for 10 min at room temperature and were then suitably diluted in the same fixative solution. All solutions were first passed through 0.45 μm membrane filters to remove particles. The results of visual and electronic counting agreed well.

3.4. Measurement of protein

Relative amounts of protein were measured in protoplasts and in the supernatant fluids from them by first growing the bacteria in CHSC medium containing [2(n)-^{3}H]-L-methionine (0.5 μCi/ml, 3.8 μmol/ml) for 4–5 generations. The protoplast suspensions prepared as described were centrifuged (12000 × *g*, 10 min), the supernatant fluids separated and the protoplasts lysed by addition of pH 6.6 phosphate buffer. Both the supernatant fluids and the lysates were mixed with equal volumes of 10% (w/v) TCA. After 30 min at room temperature the precipitates were removed on 0.45 μm membrane filters, washed successively with 0.5% TCA containing 1 mg/ml non-radioactively labelled L-methionine, and 1% (v/v) acetic acid. The membranes and precipitates were dried at 80°C overnight and their radioactivity measured by scintillation in toluene containing 0.5% 2,5 diphenyloxazole using a Beckman L.S.7800 scintillation counter.

3.5. Growth

The optical density of cultures at 675 nm wavelength was measured, the results were corrected for deviations from the Beer-Lambert Law [12]. One unit of optical density was equivalent to 0.5 mg dry weight of bacteria.

3.6. Electron microscopy

Preparations were prefixed with 3% (w/v) glutaraldehyde, 3% (v/v) formaldehyde, 0.08 M KCl and 0.01 M Mg acetate. They were then post-fixed with 1% (v/v) osmium tetroxide, prestained with 0.5% (w/v) uranyl acetate and poststained with 0.1 M Pb citrate.

4. RESULTS AND DISCUSSION

The number of protoplasts derived from exponentially growing cultures of either strain FJ6

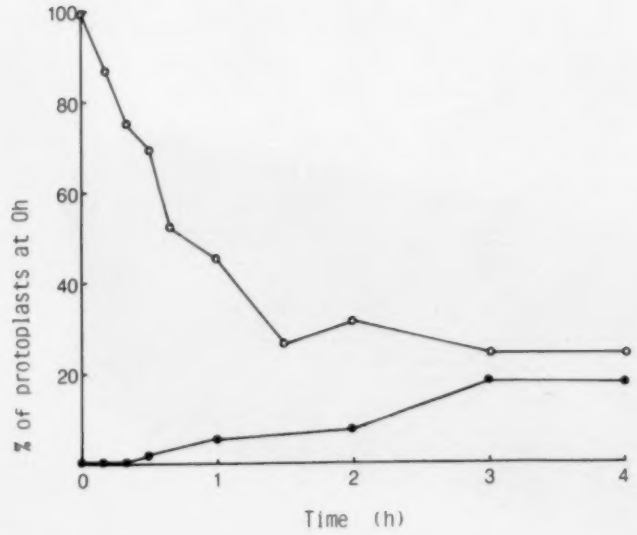
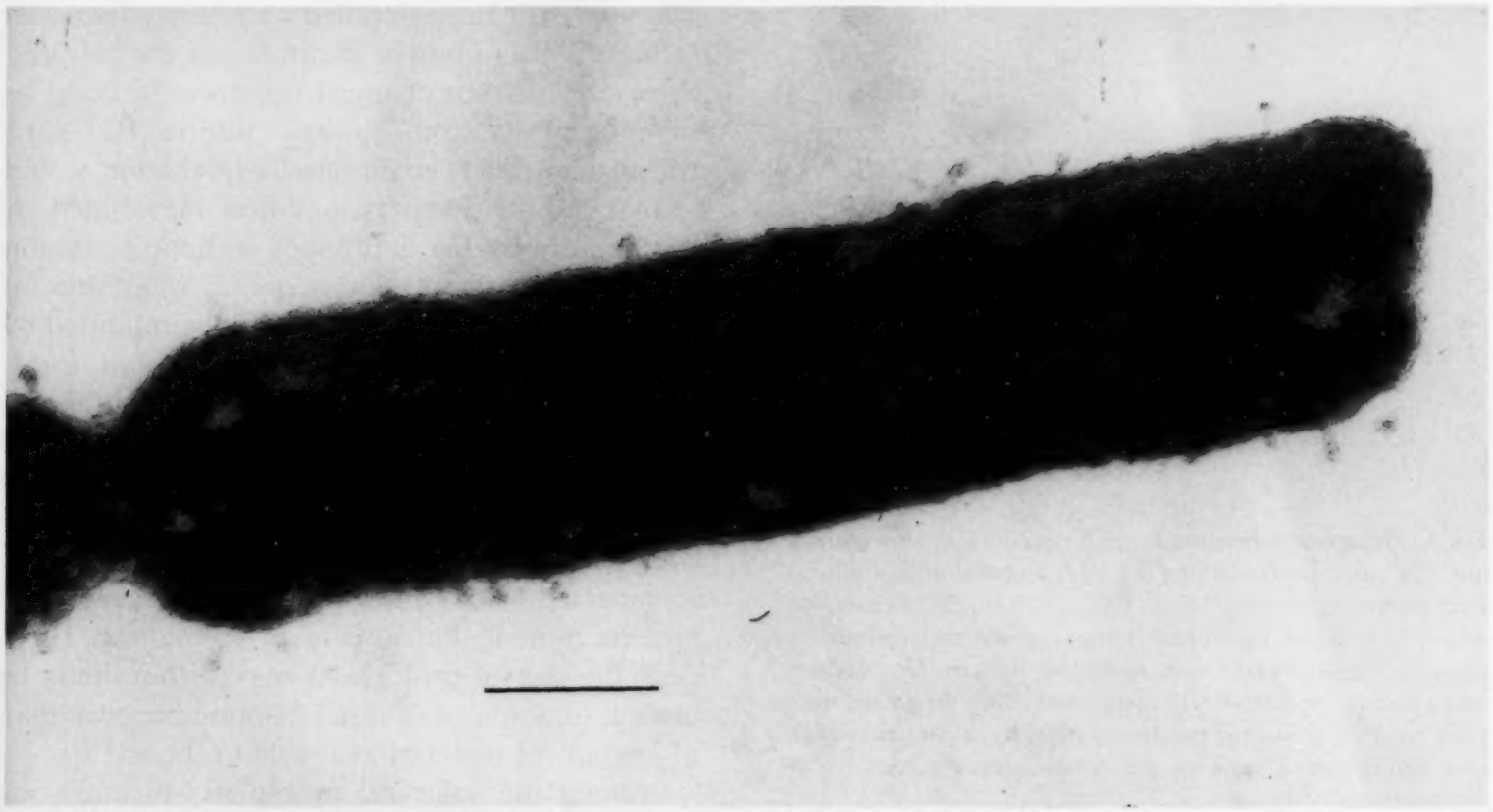


Fig. 1. Protoplast formation from *B. subtilis* FJ6 after growth in CHSC medium containing 0.2 μg/ml cephalothin. Cultures were grown exponentially to an OD_{675} of 0.15, cephalothin was added at 0 h and protoplasts were prepared and counted as described in MATERIALS AND METHODS, from culture samples taken during the next 4 h of incubation. Both the total protoplast counts (○) and the number of rods (●) present are shown as a percentage of the total protoplast count recorded before antibiotic addition.

or 168 remained constant in relation to the increasing dry weight of the bacteria. Enumeration of bacteria themselves in strain FJ6 was impossible because *B. subtilis lyt* strains grow as very long chains of unseparated cells [10] when the cultures of this strain were treated with either cephalothin (0.2 μg/ml) or methicillin (3.0 μg/ml) the number of protoplasts formed per mg bacterial dry weight fell by about 50% in the first hour of treatment. During this time the OD_{675} doubled but thereafter remained constant for at least 6 h further incubation [13]. The number of protoplasts that could be formed continued to fall until after 3–4 h it was reduced to only about 25–30% of the expected yield per mg of bacterial dry weight (see Fig. 1). It was at first thought that membrane damage had precluded protoplast formation and that lysis had occurred. If so the protein content of the supernatant fluids from protoplast preparations from antibiotic treated cultures should be higher than those from control untreated ones. This was not true. Earlier work [6] had shown that protein was not lost to the culture fluid by bacteria of FJ6 cultures treated with β-lactam antibiotics. The average protein content per protoplast had however approximately doubled (1.7 times increase) during the first hour of treatment of the cultures. Their size had not changed significantly being as for protoplasts from control cultures 1.87 ± 0.2 μm in diameter. The simplest explanation would be that cell division was inhibited immediately or very rapidly by the antibiotics without inhibition of protein synthesis. Secondary division sites of *Strep. faecalis* have been shown to be inhibited by β-lactam antibiotics [14]. Examination of transverse sections of the antibiotic treated culture by electronmicroscopy showed an increasing number of cells with an incompleted septum between two completed ones (Fig. 2b). Such cells were effectively twice the volume of normal ones and might be expected to give rise to protoplasts of twice the protein content, but also of twice the size. However, the size of protoplasts may within limits be more a function of internal osmotic pressure than of amount of material available to them [15].

Among the spherical protoplasts in antibiotic treated cultures were a number of rod shaped phase dark forms. These started to appear about 20 min, after adding the β-lactam to the cultures. Their proportion of the total was 10–15% after 1 h and up to 60% after 3–4 h (Fig. 1). If buffer not containing sucrose was gently infused under the cover-slip of a preparation on a microscope slide, the rods at first swelled to form spheres, and then burst. Examination of transverse sections under the electron microscope (Fig. 2a), confirmed the rods as membrane bounded bodies very densely packed with ribosomes. Attempts to separate rod shaped from spherical protoplasts have not been successful. Certain chemical proof of the absence from the former entities of wall material has not therefore been obtained. When the bacteria were removed from the antibiotic containing cultures and grown in CHSC without antibiotic the proportion of rod shaped protoplasts formed was reduced at the same rate as growth of the bacteria occurred suggesting that the cells yielding them were not able to multiply, to repair or to give rise to spherical protoplasts. It is interesting to note that the rod shaped protoplasts started to appear at the time that membrane damage was first detected [6] and at the time the fluorescent probe ANS started to interact with bacterial membranes



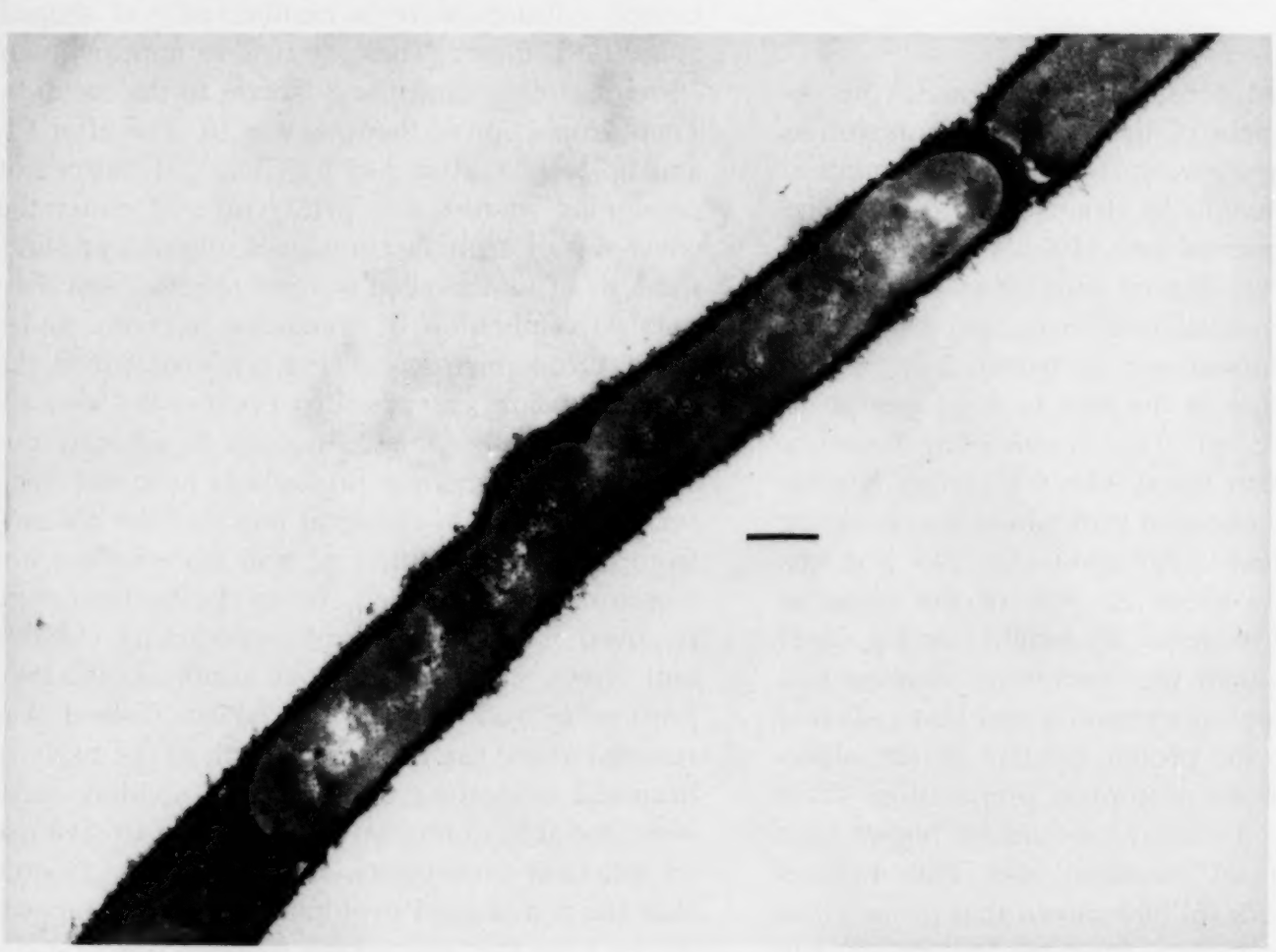


Fig. 2. (a) Transverse section of a rod-shaped protoplast, bounded by membrane only. Bar: 0.25 μm. (b) Transverse section of bacteria showing incomplete septum between two completed ones. Bar: 0.25 μm. In both (a) and (b) the culture had been treated with 0.2 μg/ml cephalothin for 3 h.

[13]. It would seem possible that the kinetics of the development of these latter phenomena were at least partly a measure of population heterogeneity with an increasing proportion of cells so badly membrane-damaged that multiplication was not possible.

Experiments with strain 168 were difficult because of the rapid lysis that occurred after adding the antibiotics to exponentially growing cultures. However, during the first hour of treatment with antibiotics when little lysis occurred rod shaped protoplasts formed about 3% of the population, and the number of total protoplasts per mg dry weight of bacteria fell to 50% of that in the control cultures. It would thus appear to the behaviour of the fully autolytic parental strain was similar to that of the *lyt* strain during the early stages of β-lactam action but cell lysis then supervened to greatly accelerate cell death.

REFERENCES

[1] Rogers, H.J., Perkins, H.R. and Ward, J.B. (1980) in Microbial Cell Walls and Membranes, pp. 298–382, Chapman and Hall, London.

[2] Tomasz, A. (1979) Ann. Rev. Microbiol. 53, 113–137.

[3] Rogers, H.J. and Frosberg, C.W. (1971) J. Bacteriol. 108, 1235–1243.

[4] McDowell, T.D. and Lemanski, C.L. (1988) J. Bacteriol. 170, 1783–1788.

[5] McDowell, T.D. and Reed, K.E. (1988) in Antibiotic Inhibition of Bacterial Cell Surface and Assembly (P. Actor, D. Daneo-Moore, M.L. Higgins, M.R.J. Salton and G.D. Shockman, eds.), pp. 231–241. ASM, Washington, D.C.

[6] Rogers, H.J., Thurman, P.T. and Burdett, I.D.J. (1983) J. Gen. Microbiol. 129, 465–478.

[7] Tomasz, A., Albino, A. and Zanati, E. (1970) Nature, (London) 227, 138–140.

[8] Shockman, G.D., Daneo-Moore, L., McDowell, T. and Wong, W. (1981) in β-lactam Antibiotics – Mode of Action, New Development and Future Prospects (Salton, M.R.J. and Shockman, G.D., eds.), pp. 31–65. Academic Press, New York and London.

[9] Horne, E., Hakenbeck, R. and Tomasz, A. (1977) J. Bacteriol. 132, 704–717.

[10] Fein, J. and Rogers, H.J. (1976) J. Bacteriol. 127, 1427–1442.

[11] Janczura, E., Perkins, H.R. and Rogers, H.J. (1961) Biochem. J. 80, 82–93.

[12] Toennies, G. and Gallant, D.L. (1949) Growth 13, 7–20.

[13] Rogers, H.J. and Wright, G. (1987) J. Gen. Microbiol. 133, 2567–2572.

[14] Higgins, M.L., Ferrero, M., and Daneo-Moore, L. (1986) J. Bacteriol. 167, 562–569.

[15] Corner, T.R. and Marquis, R.E. (1969) Biochem. Biophys. Acta 183, 544–558.

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Mapping and disruption of the *cybB* gene coding for cytochrome b_{561} in *Escherichia coli*

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1. SUMMARY

The *cybB* gene on a plasmid encoding cytochrome b_{561} in *Escherichia coli* was disrupted by insertion of Km^{rl} determinant DNA. The cromosomal *cybB* gene was replaced by the inactivated *cybB* gene on the plasmid by homologous recombination using λ phage lysogenization and heat-induction. The replacement was confirmed by Southern and Western blotting analyses. Deficiency of the *cybB* gene product did not affect the growth properties of the cells, and the oxidase activities of the cells dependent on various substrates were similar to those of the parental strain. Cytochrome b_{561} is concluded to be expressed in *E. coli*, but may not play a major role in cell growth. In the genetic map of *E. coli*, the *cybB* gene was determined by conjugational and transductional crosses to be at 31 min between *trg* and *terC*.

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Abbreviations: Km, kanamycin; Amp, ampicillin; Tet, tetracycline; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

2. INTRODUCTION

The respiratory chain of aerobically grown cells of *Escherichia coli* K12 contains six main cytochromes, which are arranged in a branched electron-carrying system [1,2]: Cytochrome b_{556}, which corresponds to a *cybA* or *sdhC* gene product in the *sdh* operon [3,4], is suggested to be a hydrophobic subunit of the succinate dehydrogenase complex. The cytochrome b_{562}-*o* complex and cytochrome b_{558}-b_{595}-*d* complex are the terminal oxidases possessing ubiquinol oxidase activities [5–8] and function as coupling sites in the aerobic respiratory chain of *E. coli* [1,2].

In addition to these predominant cytochromes, Murakami et al. [3,9] found a novel *b*-type cytochrome, cytochrome b_{561}, which is the product of the *cybB* gene, in a cytochrome b_{556} deficient mutant. This novel cytochrome was shown to be produced even in a wild-type strain [10] and was suggested to function as an intermediary electron-transferring cytochrome [9,11]. The gene for this cytochrome b_{561} was cloned on a multicopy plasmid [9], and from the cells harboring the cloned gene the cytochrome b_{561} was purified and shown to be a di-heme cytochrome *b* (α-absorption peaks at 561 and 555 nm at 77°K) with an

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oxidation-reduction potential of +20 mV [12]. The nucleotide sequence of the *cybB* gene indicated that the cytochrome b_{561} is a polypeptide of 175 amino acids with a molecular weight of 20160 and it is predicted to be a very hydrophobic protoheme-protein with three membrane-spanning regions [13]. However, the physiological role of this cytochrome is not yet known.

In this study, we have disrupted the *cybB* gene in the *E. coli* chromosome, mapped the gene, and examined the effect of deficiency of this cytochrome on the growth and oxidase activities of the cells.

3. MATERIALS AND METHODS

3.1. Strains and plasmids

The strains and plasmids used are listed in Table 1. pHI 59 was obtained from Dr. H. Inouye,

Table 1

Bacterial strains, phages, and plasmids used

Strain	Relevant genotype	Source
KL16	Hfr	Bachmann (CGSC strain)
ET1214	*thi zig*-219::Tn*10*	Bachmann (CGSC strain)
p3478	*thyA polA deoC θ(rrnD-rrnE)*	Bachmann (CGSC strain)
ST501	same as p3478 except *zig-219*::Tn*10*	Kajie, S. Tet^r transductant of p3478 with $P1_{kc}$ grown on ET1214
ST503	same as KL16 except *zig-219*::Tn*10 polA*	Kajie, S. Tet^r tranductant of KL16 with $P1_{kc}$ grown on ST501
LE392	*hsdR514 supE44 supF58*	Enquist [15]
ST1589	same as ST503 except λ_{cyby}	λ_{cyby} lysogen of ST503
ST1597	same as ST1589 except λ^- Km^r	ts^r, Km^r isolate of ST1589
JRG861a	*fnr-1 gal trpA trpR rpsL*	Guest, J.R. [26]
TH589	Hfr(KL99) *pro*::Tn*5 thi trg-2*::Tn*10*	Harayama, S.
ST1604	same as JRG861a except *trg-2*::Tn*10*	Tet^r transductant of JRG861a with $P1_{kc}$ grown on TH589
TK2063	Δ(gal-bio) *trkA405 trkD1 thi rha*	Roads et al. [4]
TK3D11	Δ(*kdp-glt*A-*sdh*)*D-01* Δ(*gal-bio*) *trkA405 trkD1 th rha*	Roads et al. [4]
ST1609	same as TK3D11 except Km^r	Km^r transductant of TK3D11 with $P1_{kc}$ grown on ST1597
ST1613	same as TK2063 except Km^r	Km^r transductant of TK2063 with $P1_{kc}$ grown on ST1597
TH307	*thi his tyrA purC trp lac gal xyl mtl malA rpsL tonA tsx*	Harayama, S.
IY12	*thi his ilv trp rpsL ndh*	Young, I. [25]

Plasmid	Comment	Source
pAM5167	Amp^r, *cybB*$^+$	Murakumi et al. [9]
pH159	Km^r, Tet^r	Inoue, H.
pUME53	same as pAM5167 except having Km^r determinant in *cybB* gene	This study
pUME531	same as pUME53 except having *Sal*I linker at *Hin*cII site	This study

Phage	Comment	Source
$\lambda_{wes}\lambda_B$	Wam403 Eam1100 Sam cI857	Tiemeier et al. [15]
λ_{cyby}	$\lambda_{wes}\lambda_B$ having inserted pUME531 at *Sal*I site	This study

and has Km^r determinant DNA at the pBR322 *Eco*RI site flanked by inverted repeats of the multilinker sequence of pUC4K [14] with the addition of an *Xba*I site between the *Eco*RI and *Sal*I sites.

The disruption of the *cybB* gene on the *E. coli* chromosome is described in the legend to Fig. 1.

3.2. DNA manipulation

Restriction endonuclease digestion, DNA isolation, ligation, analysis by agarose gel electrophoresis and in vitro λ phage packaging were performed as described in [15].

Chromosomal DNA was obtained as described in [9]. Southern blotting analysis was done as in [15], using pAM5167 labeled with [α-^{32}P]dATP by nick-translation procedure as a probe.

3.3. Genetic procedures

Conjugations of cells and transductions with P1 phages were performed as described in [16]. λ Phage sensitivity was examined by the cross-streaking method. Cells were grown in LB medium [15] or DM minimal medium [17] supplemented with amino acids (100 μg/ml) and vitamins (1 μg/ml) when required. Carbon sources were added at final concentrations of 0.4%. The phenotype of *pol*A was examined by measuring methylmethane sulfonate sensitivity [18].

3.4. Immunological detection of cytochrome b_{561} *in membranes from various strains*

Cells grown in LB medium (5 ml) were harvested at a density of about 6×10^8 cells/ml and membranes were obtained by a published procedure [19]. The membranes were subjected to SDS-PAGE, and the gels were electrotransferred to nitrocellulose filters by a reported method [20]. Cytochrome b_{561} was detected on the filter using specific antiserum against cytochrome b_{561} obtained by Murakami et al. from rabbits immunized with purified cytochrome b_{561} [12] by a reported procedure [20].

3.5. Measurement of oxidase activity of cells and membranes

Oxidase activities in cells and membranes in the presence of various respiratory substrates were measured as described [21]. Proteins were determined by a standard procedure [22], with bovine serum albumin as a standard.

3.6. Enzymes and chemicals

[α-^{32}P]dATP (> 3000 Ci/mmol) and [^{125}I] protein A (100 mCi/mmol) were obtained from Amersham. A nick-translation kit was also obtained from Amersham. Restriction enzymes and T_4 DNA polymerase were purchased from Takara Shuzo Co., Kyoto, Japan. *Sal*I linker was obtained from New England Biolab. Other chemicals were commercial products of analytical grade.

4. RESULTS

4.1. Disruption of the cybB *gene on the* E. coli *chromosome*

Since we have a clone for the *cybB* gene (9, 13), disruption of the gene on the chromosome was possible (Fig. 1).

The disruption of the *cybB* gene was confirmed by Southern blotting analyses of the DNAs from various strains and Western blotting analyses of the membranes. As shown in Fig. 2A, the lysogen ST1589 had a *Sal*I fragment (about 7 kb) which was bigger than the *Sal*I fragment from wild type cells (about 6 kb) and it also had *Sal*I fragments derived from pUME531. After heat-induction, strain ST1597 had lost the original *Sal*I fragment containing the *cybB* gene and the fragments from pUME531. Instead this strain retained the bigger *Sal*I fragment resulting from replacement of the chromosomal *cybB* gene by the disrupted *cybB* gene on the plasmid. The immunological detection of cytochrome b_{561} in membranes using specific antiserum also confirmed the absence of cytochrome b_{561} in strain ST1597 membranes, as shown in Fig. 2B.

4.2. Genetic mapping of the cybB *gene*

Km^r determinant DNA was inserted into the chromosomal *cybB* gene, as described in the previous section. Therefore, the *cybB* gene was mapped using Km^r as a marker.

Conjugational crosses of strains JRG861a and TH307 with ST1597 suggested that the Km^r gene

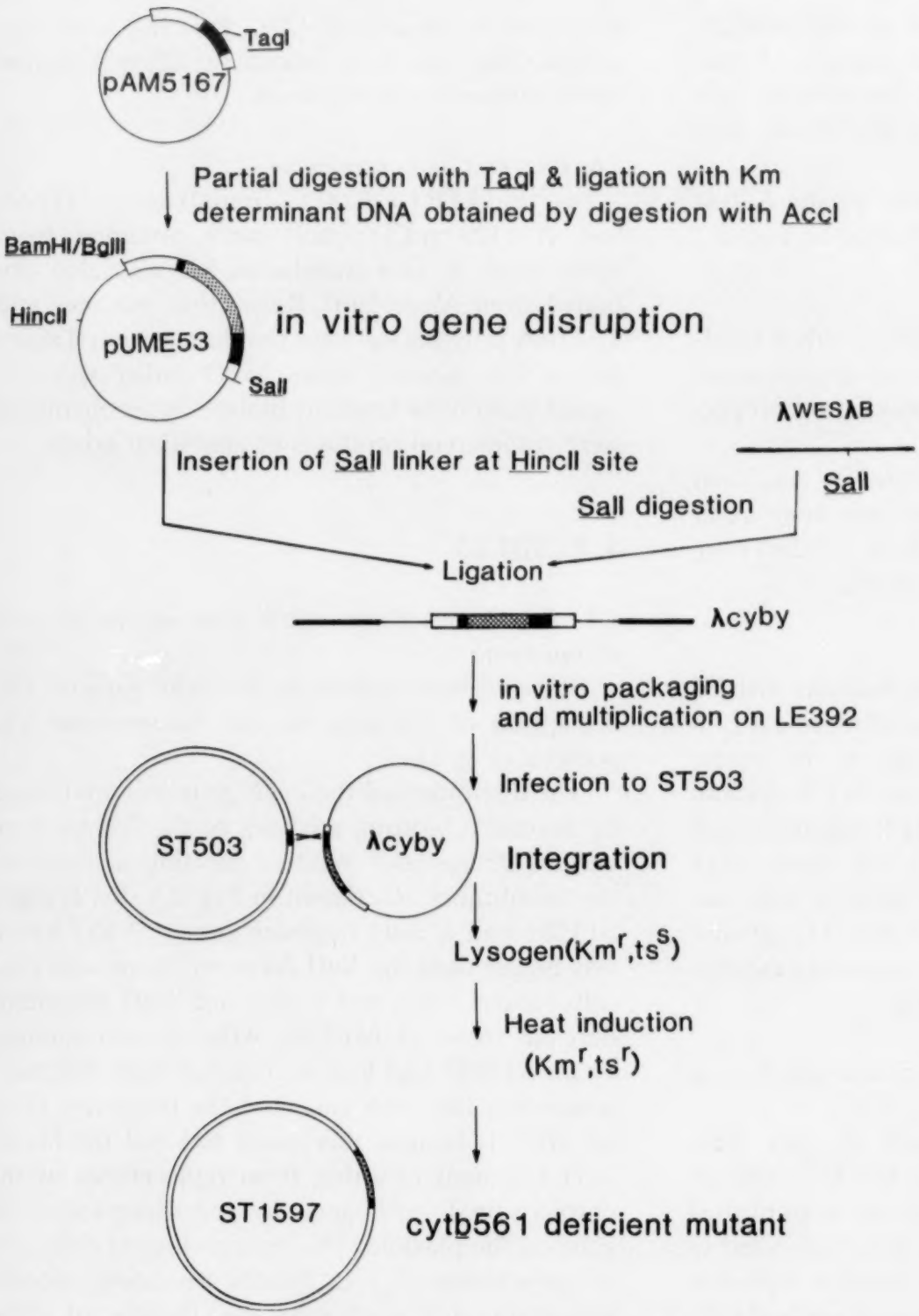

Fig. 1. Disruption of the *cybB* gene on the *E. coli* chromosome. Plasmid pUME53 was constructed by inserting Km^r determinant DNA, derived from pHI59 digested with *Acc*I, into a unique *Taq*I site within the *cybB* gene in pAM5167 [9]. The direction of transcript of the kanamycin inactivation enzyme was shown to be the opposite of that of the *cybB* gene by examining the direction of the DNA sequence by restriction endonuclease digestion [13]. pUME53 was digested with *Hin*cII, to which a synthetic *Sal*I linker was ligated, and pUME531, having the *Sal*I site inserted at the *Hin*cII site in the Amp^r gene in pBR322, was constructed. pUME531 and λwesλB were digested with *Sal*I and ligated. Then the mixture was in vitro packaged and infected into strain LE392. After propagation of the phages, the plates were replicated onto LB plates containing 50 μg/ml kanamycin, and kanamycin resistant lysogens were selected at 30 °C. The phages from these Km^r isolates were induced by heat treatment, and the DNAs of these phages were obtained as described elsewhere [15]. A DNA of these phages was shown to have an insert of intact pUME531 digested at only one *Sal*I site into the λwesλB *Sal*I cloning site, suggesting that this phage can also exist as an episome owing to the pBR322 *ori* activity. Therefore, the phage was infected into ST503 (polA strain) and Km^r lysogens were obtained, which were also λcI resistant. From these lysogens, Km^r and temperature-resistant isolates were obtained on LB plates containing glucose and kanamycin incubated at 42 °C. The strains obtained were confirmed to be λcI sensitive.

was located for *fnr*, proximal to *his*. Then strain ST1604 was constructed and this strain was transduced with $P1_{kc}$ phage grown on ST1597. Among 202 Km^r colonies selected, 191 were Tet sensitive and 2 were fnr^+. Furthermore, all the fnr^+ transductants were Tet sensitive, suggesting that the Km^r gene is located close to *trg* and distal to *fnr*, as shown in Fig. 3.

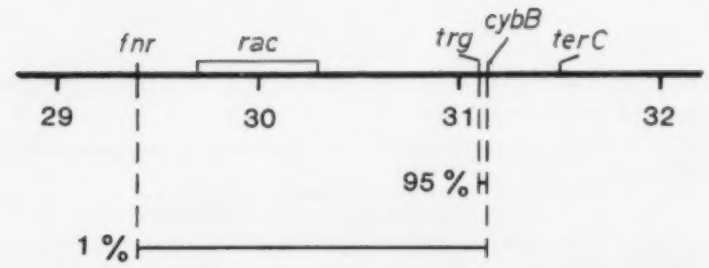

Fig. 3. Mapping of the *cybB* gene on the *E. coli* chromosome. The thick line and the numbers below it indicate the *E. coli* chromosome and map positions in min. Strain ST1604 (*fnr-1*, *trg-2*::Tn*10*) was transduced with $P1_{kc}$ phage grown on ST1597 (*cybB*::Km^r) and Km^r transductants were selected. Of these Km^r transductants, 95% (191/202) were Tet^s and 1% (2/202) were fnr^+, showing that the map location of the *cybB* gene was as indicated in the figure.

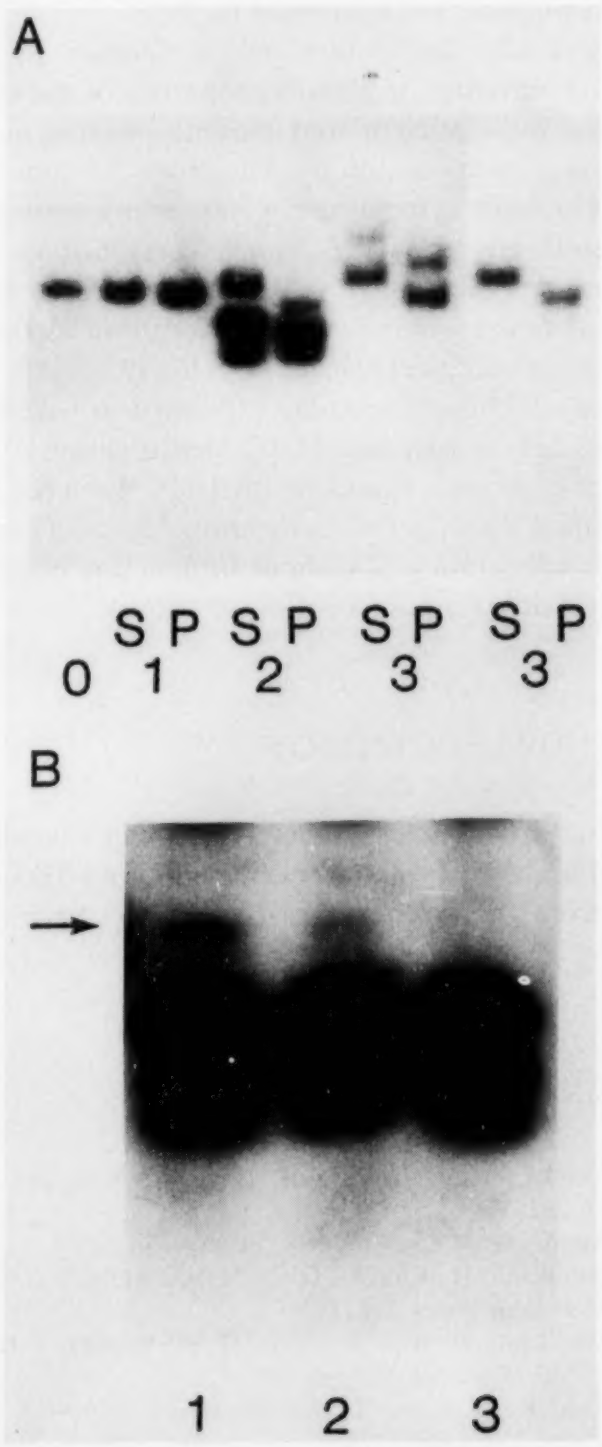

Fig. 2. Southern (A) and Western (B) blotting analyses of wild type and *cybB* deficient strains.
(A) Chromosomal DNAs were obtained from stationary phase cells of LE392 (lane 0), ST503 (lane 1), ST1589 (lane 2), and ST1597 (lane 3) as described in MATERIALS AND METHODS. The DNA from LE392 was digested with *Sal*I only and the DNAs from other strains were digested with *Sal*I (S) or *Sal*I/*Pst*I (P). The digests were subjected to Southern blotting analysis using pAM5167 as a hybridization probe. Lane 3 is shown in duplicate. Since the left pair of lane 3 were the results due to the incomplete digestion of DNA by restriction enzymes, results from the same experiments after complete digestion were shown in the right pair of lane 3.
(B) Crude membranes obtained from ST503 (1), ST1589 (2) and ST1597 (3) as described in MATERIALS AND METHODS were subjected to Western blotting analysis according to the described procedure using specific antiserum against purified cytochrome b_{561} obtained from immunized rabbits. The arrow indicates the position of cytochrome b_{561} protein determined by running the purified cytochrome in parallel.

4.3. Effect of cytochrome b_{561} *deficiency on growth properties and oxidase activities*

Cytochrome b_{561} was suggested to be an intermediary electron transferring component in the aerobic respiratory chain of *E. coli* [9,11]. We examined the effects of deficiency of this cytochrome on the physiology, and especially the oxidase activities of strains ST503 and ST1597, and the cytochrome b_{556}-deficient strains TK3D11 and ST1609 (Table 2). Essentially no difference was found between the cytochrome b_{561} deficient strain and the parental strain. Similar results were obtained with the other respiratory substrates ex-

Table 2

Oxidase activities of sonicates from strains with wild type and mutated cytochrome b_{561}

Substrate	Strain	
	TK3D11	ST1609
NADH	172 [a]	130
L-Lactate	67	55
Pyruvate	21	22
Malate	10	12

[a] nmol O_2/min/mg protein.

amined, D-lactate, glycerol-3-phosphate, formate, oxaloacetate, NADPH, succinate and deamino NADH.

Consistent with these results on oxidase activities, the growths of strain ST1609 on various carbon sources were similar to those of the parental strain.

5. DISCUSSION

Insertion inactivation of the *cybB* gene on the *E. coli* chromosome was achieved by a two-step procedure: 1) Disruption of the *cybB* gene on a plasmid by insertion of Km^r determinant DNA, and 2) replacement of the wild-type *cybB* gene by the disrupted *cybB* gene on the plasmid by in vivo recombination using phage integration and heat-induction. During construction of the hybrid λ phage, we failed to obtain a phage with the *Sal*I fragment containing the *cybB* gene, and instead obtained a hybrid of the λ phage and the plasmid itself ligated at the *Sal*I site. We do not known the reason for this failure, but to obtain true lysogenization of the phage-plasmid hybrid, we used strain ST503 (*polA*) as a host.

The *cybB* gene was located close to the *trg* gene by genetic mapping. This region has only a few genetic markers, but the DNA around this region has already been cloned [23,24] and its restriction map has been reported. We found that although the restriction maps did not correspond completely with the restriction sites of the *cybB* gene [9,13] partial homology of the restriction sites of *Hin*dIII, *Eco*RI and *Pst*I were discernible in the region from 1500 to 1540 kb in Fig. 6 of the published paper [24].

Although we raised the antiserum against the purified cytochrome b_{561} [12], the serum cross-reacted with a few polypeptides in membranes on Western blotting. However, as long as the immunodetection of cytochrome b_{561} is concerned, the serum could identify it unequivocally in Western blotting analysis. In addition, we have noticed that some polypeptides with higher molecular weights were missing concomitantly with the cytochrome b_{561} in strain ST1597 (data not shown), which we have not examined in detail.

We could not detect any difference in the oxidase activities or growth properties of the cytochrome b_{561}-deficient and parental strains under various growth conditions. Therefore, we conclude that although cytochrome b_{561} is really expressed in aerobically grown *E. coli*, it may not be important for the growth or physiology of the cells. If its deficiency results in a defect in an auxiliary process in cell metabolism, as is likely because the amount of this cytochrome expressed in wild-type *E. coli* cells is very small [10], identification of the defective process would be difficult. We have also examined the effect of cytochrome b_{561} deficiency in the *ndh* strain [25] without finding any essential abnormality (unpublished observation).

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REFERENCES

[1] Anraku, Y. and Gennis, R.B. (1987) Trends Biochem. Sci. 12, 262–266.
[2] Anraku, Y. (1988) Ann. Rev. Biochem. in press.
[3] Murakami, H., Kita, K., Ohya, H. and Anraku, Y. (1984) Mol. Gen. Genet. 196, 1–5.
[4] Murakami, H., Kita, K., Ohya, H. and Anraku, Y. (1986) FEMS Microbiol. Lett. 30, 307–311.
[5] Kita, K., Konishi, K. and Anraku, Y. (1984) J. Biol. Chem. 259, 3368–3374.

[6] Kita, K., Konishi, K. and Anraku, Y. (1984) J. Biol. Chem. 259, 3375–3381.
[7] Lorence, R.M., Koland, J.G. and Gennis, R.B. (1986) Biochemistry 25, 2314–2321.
[8] Hata-Tanaka, A., Matsuura, K., Itoh, S. and Anraku, Y. (1987) Biochim. Biophys. Acta 893, 289–295.
[9] Murakami, H., Kita, K. and Anraku, Y. (1984) Mol. Gen. Genet. 198, 1–6.
[10] Kita, K., Murakami, H., Ohya, H. and Anraku, Y. (1985) Biochem. Int. 10, 319–326.
[11] Withers, H.K. and Bragg, P.D. (1987) Biochim. Biophys. Acta 892, 10–22.
[12] Murakami, H., Kita, K. and Anraku, Y. (1986) J. Biol. Chem. 261, 548–551.
[13] Nakamura, H., Murakami, H., Yamato, I. and Anraku, Y. (1988) Mol. Gen. Genet. 212, 1–5.
[14] Vieira, J. and Messing, J. (1982) Gene 19, 259–268.
[15] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
[16] Mogi, T., Yamamoto, H., Nakao, T., Yamato, I. and Anraku, Y. (1986) Mol. Gen. Genet. 202, 35–41.
[17] Davis, B.D. and Mingioli, E.S. (1959) J. Bacteriol. 60, 17–28.
[18] DeLucia, P. and Cairns, J. (1969) Nature 224, 1164–1166.
[19] Yamato, I., Anruka, Y. and Ohki, M. (1979) J. Biol. Chem. 254, 8584–8589.
[20] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA, 76, 4350–4354.
[21] Kasahara, M. and Anraku, Y. (1974) J. Biochem. 76, 967–976.
[22] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[23] Bouche, J.P. (1982) J. Mol. Biol. 154, 1–20.
[24] Kohara, Y., Akiyama, K. and Isono, K. (1987) Cell 50, 495–508.
[25] Young, I.Y. and Wallace, B.J. (1976) Biochim. Biophys. Acta 449, 376–385.
[26] Shaw, D.J. and Guest, J.R. (1981) Mol. Gen. Genet. 181, 95–100.

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Thymidine phosphorylase and uridine phosphorylase of *Lactobacillus casei*

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1. SUMMARY

Crude extracts of *Lactobacillus casei*, were found to possess both thymidine phosphorylase and uridine phosphorylase activities, which were associated with a single sucrose gradient band. However, using dye ligand chromatography (Green A), two enzymatic fractions were obtained each of which possessed mainly one of these activities. By this procedure, the uridine phosphorylase was purified 329-fold and the thymidine phosphorylase about 25-fold. The existence of the two phosphorylases was supported by K_m, K_i, pH optimum, and heat stability studies. Identical bands were obtained on sucrose gradient sedimentation for the purified enzymes and for their mixture in a crude extract, suggesting that they have similar sedimentation coefficients, and do not form an aggregate in the crude extract.

2. INTRODUCTION

The enzymes which catalyze the phosphorolysis of pyrimidine nucleosides are involved in the degradation of these compounds and in their utilization as energy sources, or in the rescue of pyrimidine bases for nucleoside synthesis. They carry out the following reactions:

Thymidine + phosphate
⇄ thymine + deoxyribose-1-phosphate

Uridine + phosphate
⇄ uracil + ribose-1-phosphate

Escherichia coli and *Salmonella typhimurium* possess a thymidine phosphorylase (EC 2.4.2.4) [1–6] which acts with deoxyribose nucleosides, and a uridine phosphorylase (EC 2.4.2.3) [5,7–9] which is specific for uracil ribonucleosides. The uridine phosphorylase of *E.coli* K_{12} also shows some activity with deoxyuridine and thymidine [7]. It was reported that *Bacillus stearothermophilus, Haemophilus influenzae* and possibly *B. subtilis*, contain one enzyme, pyrimidine nucleoside phosphorylase, (EC 2.4.2.2) [10,11], which acts with both thymidine and uridine.

Earlier attempts to demonstrate enzymatic phosphorolysis of these substrates in lactobacilli have failed [12]. Unlike most bacteria, lactobacilli possess a trans-*N*-deoxyribosylase (trans-*N*-glycosidase, EC 2.4.2.6) which catalyzes a phosphate independent direct transfer of deoxyribose moieties between a wide variety of purines and

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pyrimidines [13,14]. This observation supported the assumption that they were devoid of pyrimidine phosphorylases. However, in 1974, a thymidine phosphorylase activity was found in *Lactobacillus acidophilus* [15–16]. We have recently observed a similar activity in *Lactobacillus casei* [17].

In this paper we characterize both the thymidine and the uridine phosphorylase activities of *L. casei* and provide evidence for their association with two separable proteins.

3. MATERIALS AND METHODS

3.1. Bacterial strains

The strains used were: *L. casei* ATCC No 7469, *E. coli B* ATCC No 11303 and *B. subtilis* ATCC No 6633.

3.2. Media and bacterial growth conditions

L. casei was grown in a modified 'Folic acid casei medium' (Difco), which contained 1% glucose, 200 pg/ml folic acid and lacked sodium acetate. Erlenmyer flasks (250 ml) containing 200 ml medium were inoculated with 1 ml of a 24 h culture, and incubated overnight at 37 °C without aeration. Growth was measured in a Coleman Junior Spectrophotometer at 600 nm and the cells were harvested at the logarithmic phase (0.3 O.D.). The cultures were neutralized with a NaOH solution and the cells were spun down at $34{,}540 \times g$ for 30 min at 10 °C. The cells were washed twice with an equal volume of 0.025 M Tris buffer (pH 7.0). *E. coli* and *B. subtilis* cells were grown with shaking in the modified *L. casei* medium at 37 °C, were spun down by centrifugation at $12\,000 \times g$ for 5 min in the cold, and washed as indicated above.

3.3. Preparation of enzyme extracts

The cells were suspended in 0.05 M Tris buffer (pH 7.0) containing 5 mM 2-mercaptoethanol and 2 mM EDTA (about 7×10^9 cell/ml) and disrupted in the cold by low (*L. casei*) or high (*E. coli* and *B. subtilis*) power sonication in a MSE sonifier, for a total of 55 s with two intermissions of 10 s each. Cell debris were removed by centrifugation at $34\,540 \times g$ for 30 min at 10 °C.

3.4. Assay of enzyme activity

Thymidine phosphorylase and uridine phosphorylase activities of the extracts were assayed by measuring the amount of thymine or uracil formed from thymidine or uridine, respectively, using the method of Razzell and Khorana [2]. An enzyme unit was defined as the amount of enzyme which catalyzed the formation of lumole thymine or uracil in one minute at the assay conditions.

The reaction mixture for the assay of the activity of the phosphorylases contained: thymidine or uridine 10 mM, EDTA 0.25 mM, 2-mercaptoethanol 5 mM, phosphate buffer 200 mM (pH 6.0 for thymidine phosphorylase, pH 7.0 for uridine phosphorylase) and about 2 mg/ml cell extract protein. The enzymatic reactions were carried out at 37 °C for 30 min and stopped by adding NaOH to a final concentration of 0.5 M. The absorbance of the assay mixture was read at 300 nm (for thymidine phosphorylase) or 290 nm (for uridine phosphorylase), using a Spectronic 21 spectrophotometer. For calculations, the following constants were used: conversion of 1 mM thymidine to 1 mM thymine $\Delta A_{300} = 3.4$, conversion of 1 mM uridine to 1 mM uracil $\Delta A_{290} = 5.4$. Microamounts of protein were determined by a modification of the Lowry method [18].

3.5. Sucrose density gradient centrifugation

Samples of 0.2 ml of *L. casei* extract (3 mg protein) were loaded on 5 ml of linear 5–20% sucrose gradients and were centrifuged for 18 h at $210\,000 \times g$. Fractions of 0.5 ml were collected and assayed for thimidine phosphorylase and uridine phosphorylase activities.

3.6. Purification by dye ligand chromatography [19]

The fractionation was carried out at room temperature. Small columns containing 2 ml dye gel matrix (Amicon Co.) were equilibrated with Buffer A, which contained 20 mM Tris-HCl buffer (pH 7.5) and 5 mM 2-mercaptoethanol. A volume of 0.5 ml crude extract (7.5 mg protein) was loaded on each column. After 30 min the columns were washed with 10 ml of Buffer A and eluted with 10

ml of the same buffer containing 2 mM thymidine or uridine. The proteins which remained on the column were eluted with Buffer A, which contained 1.5 M KCl.

4. RESULTS AND DISCUSSION

4.1. Characterization of the phosphorolysis activities of L. casei *extracts with thymidine or uridine as substrates*

Due to the lability of the purified preparations we had to carry out most of our experiments with crude extracts. Significant differences between the properties of the indicated phosphorolysis activities were observed.

4.1.1. pH optimum. Maximal activity with thymidine as substrate was obtained at pH 6.0, while the maximal activity with uridine was at pH 7.0.

4.1.2. K_m and inhibitors. The K_m values for thymidine, uridine and phosphate were 1.66×10^{-3} M, 3.8×10^{-3} M and 1×10^{-1} M, respectively. The effect of various compounds on the activities with either thymidine or uridine is presented in Table 1. 5-Bromo-2-deoxyuridine inhibited only the activity with thymidine, while thymidine-5-monophosphate and 3-O-methyl-α-D-glucopyranoside inhibited only the activity with uridine. Both activities were inhibited by 5-fluoro-2-deoxyuridine. A higher concentration of 6-methyluracil was required for the inhibition of the thymidine activity than for the uridine one. 2-Deoxyribose-1-phosphate and 2-deoxyuridine-5-monophosphate did not have any inhibitory effect at the concentration of 10 mM. The inhibition constants (K_i) of 6-methyluracil for the activities with thymidine or uridine were 1.6×10^{-3} M and 1.5×10^{-4} M, respectively. In both cases the inhibition was competitive. The inhibition constant of 3-O-methyl-α-D-glucopyranoside for the activity with uridine was 1.4×10^{-3} M.

Table 1

Inhibition of phosphorylase activities of *L. casei* extracts with thymidine or uridine by various compounds

Compound [a]	Concentration (mM)	% Inhibition of [b] activities	
		with thymidine	with uridine
5-Bromo-2-deoxyuridine	10	80	0
Thymidine-5-monophosphate	10	0	100
3-0-Methyl-α-D-glucopyranoside	10	0	100
5-fluorodeoxyuridine	10	100	88
6-methyluracil	10	86	100
6-methyluracil	1	36	100

[a] The enzymatic activities in the presence of the indicated compounds, were assayed as described under MATERIALS AND METHODS.

[b] The concentration of the substrate was 10 mM.

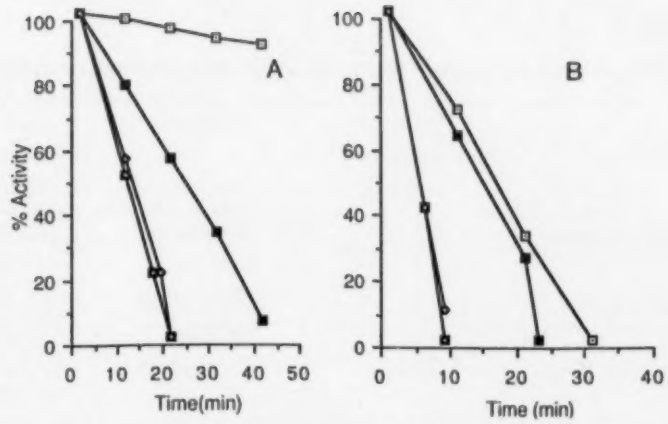

Fig. 1. Heat stability of the activities of (A) *L. casei* and (B) *E. coli* extracts with thymidine or uridine. The extracts were incubated at 55 °C or 60 °C. At various periods of time, samples were taken out and assayed for the activity with thymidine or uridine as described under MATERIALS AND METHODS. Activity with thymidine after incubation at 55 °C (⊡) and at 60 °C (◇). Activity with uridine after incubation at 55 °C (■) and 60 °C (□).

4.1.3 Heat stability. Fig. 1 shows that after incubation at 60 °C both activities of *L. casei* extracts were lost within about 20 min. However, after incubation at 55 °C for 40 min the activity with thymidine was reduced by 10% while that with uridine by 95%: The *E. coli* extracts lost their activity with thymidine or uridine at either 55 °C or 60 °C within about 30 min and 10 min, respectively.

4.2. Sucrose density gradient centrifugation studies

To find out if *L. casei* extracts contain one enzyme with two activities, a pyrimidine nucleoside phosphorylase, or two separate enzymes, thymidine phosphorylase and uridine phosphory-

Table 2

Purification of thymidine phosphorylase and uridine phosphorylase of *L. casei* by dye ligand chromatography [a]

	Total protein (mg)	Substrate	Total activity (units)	Specific activity [b]	Recovery (%)	Purification (fold)
Crude extract	7.5	Thymidine	250	33	100	1
		Uridine	100	13	100	1
Fraction A	4.4	Thymidine	26	6	10	0.18
		Uridine	29	6.6	29	0.5
Fraction B	0.018	Thymidine	2	111	0.8	33
		Uridine	77	4278	77	329
Fraction C	0.21	Thymidine	175	833	70	25.3
		Uridine	2	95	2	0.7

[a] The details of the purification procedure are described under MATERIALS AND METHODS. The fractionation chart is presented in Fig. 3.

[b] Units per mg protein.

lase, they were subjected to sucrose gradient centrifugation. For comparison, we analyzed the extracts of *E. coli* and *B. subtilis*. Fig. 2 shows that extracts of *L. casei* and *B. subtilis* gave single peaks, which possessed both activities, while the *E. coli* extract yielded two distinctly separated peaks, one of which was active mainly with uridine and the other mainly with thymidine. These results suggested that the *L. casei* extract contains a single pyrimidine nucleoside phosphorylase, as was reported for *B. stearothermophilus, H. influenzae* and *B. subtilis* [10,11]. However, this assumption was disproved by the results of the purification experiments.

4.3. *Purification of the phosphorylases of* L. casei *and* E. coli *by dye ligand chromatography*

By the use of dye ligand (Green A) chromatography [19] we succeeded to obtain two purified enzymatic fractions which showed specificity towards thymidine or uridine. Fig. 3 shows that the fraction eluted by the Tris-thymidine solution (B)

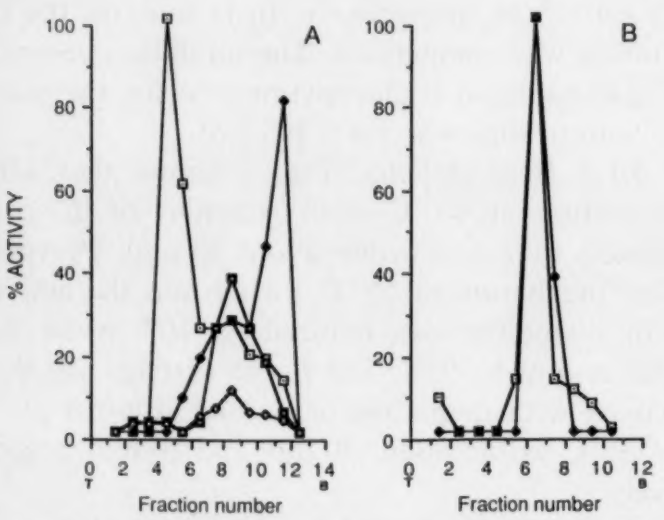

Fig. 2. Sucrose density gradient centrifugation of (A) *E. coli* and *L. casei* extracts and (B) of a *B. subtilis* extract. The details of the procedure were described under MATERIALS AND METHODS. The fractions were collected from top (T) to bottom (B). Activity with thymidine of extracts of *E. coli* or *B. subtilis* (⊡), and of *L. casei* (□). Activity with uridine of extracts of *E. coli* or *B. subtilis* (■) and of *L. casei* (◇).

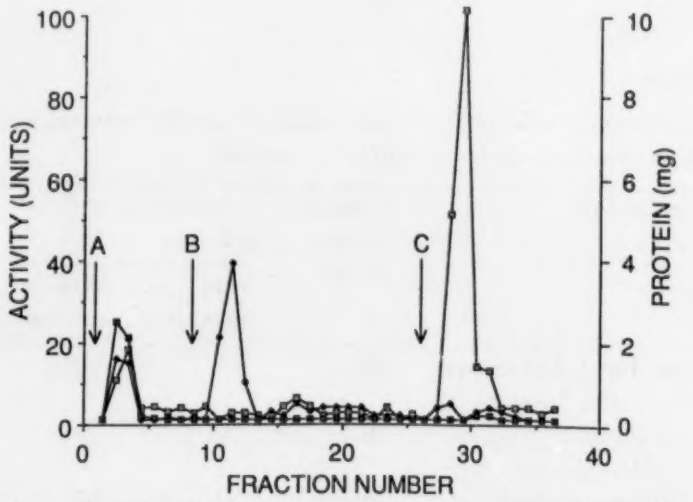

Fig. 3. Dye ligand chromatography of *L. casei* extracts. The extracts were loaded on a Green A column and eluted as described under MATERIALS AND METHODS. At the points indicated by the vertical arrows the elution with the following solutions was started: (A) Buffer A, (B) Buffer A containing 2 mM thymidine (C) Buffer A containing 1.5 M KCl. Each fraction contained 1 ml. Activity with thymidine (⊡), with uridine (■). Protein (□).

has a high specificity for uridine. Subsequent elution with Tris-1.5 M KCl yielded another fraction (C) which was active mainly with thymidine. Fraction A may represent the excess enzyme which did not bind to the column. Table 2 shows that the recovery of the uridine phosphorylase activity was 77% and it was purified 329-fold. The thymidine phosphorylase was purified about 25-fold with a recovery of 70%. Unlike the *L. casei* thymidine phosphorylase, the *E. coli* enzyme practically did not adhere to the Green A column. However, its uridine phosphorylase could be eluted by 2 mM uridine, as was reported before [19], and also by 2 mM thymidine.

4.4. Sucrose density gradient centrifugation of the purified fractions

To reconcile the apparent discrepancy between the finding of two phosphorylysis activities with one band on the sucrose gradient analysis of the crude extract, and its separation by the dye-ligand chromatography into two fractions, each of which contained only one activity, the following possibilities were considered: (a) both enzymes have a similar sedimentation coefficient on the sucrose gradient, (b) the two enzymes form an aggregate, which runs as a single peak on the sucrose gradient centrifugation and can be split under proper conditions. The observation (Fig. 4) that each of the purified phosphorylases as well as their mixture in a crude extract sedimented at the same rate on the sucrose gradient favours the first possibility.

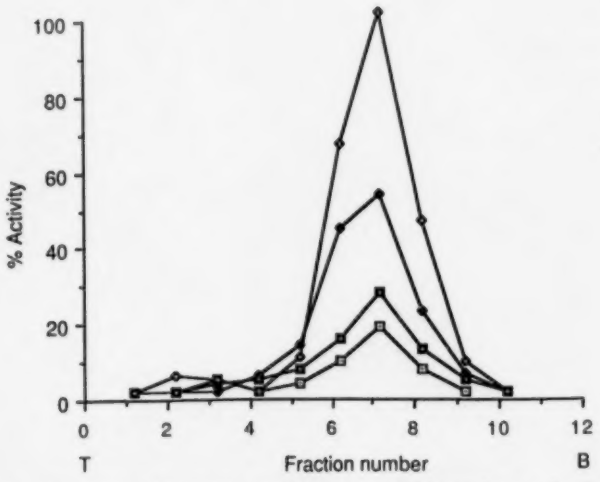

Fig. 4. Sucrose density gradient centrifugation of purified fractions and of a crude extract of *L. casei*. The details of the procedure are described under MATERIALS AND METHODS. The fractions were collected from top (T) to bottom (B). Activity of purified thymidine phosphorylase (□), of purified uridine phosphorylase (⊡). Activity of crude extract with thymidine (◇) with uridine (■).

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REFERENCES

[1] Manson, L.A. and Lampen, J.O. (1951) J. Biol. Chem. 193, 539–547.
[2] Razzel, W.E. and Khorana, H.G. (1958) Biochim. Biophys. Acta 28, 562–566.
[3] Razzel, W.E. and Casshyap, P.J. (1964) J. Biol. Chem. 239, 1789–1793.
[4] Schwartz, M. (1971) Eur. J. Biochem. 21, 191–198.
[5] Beck, C.F., Ingraham, J.L. and Neuhard, J. (1972) J. Bacteriol. 110, 219–226.
[6] Hoffee, P.A. and Blank, J. (1978) in Methods in Enzymology (Collowick, S.P. and Kaplan, N.O., eds.), Vol. 51, pp. 437–442, Academic Press, New York.
[7] Leer, J.C., Hammer Jespersen, K. and Schwartz, M. (1977) Eur. J. Biochem. 75, 217–224.
[8] Krenitsky, T.A. (1976) Biochim. Biophys. Acta 429, 352–358.
[9] Munch-Petersen, A. (1968) Eur. J. Biochem. 6, 432–442.
[10] Saunders, P.P., Wilson, B.A. and Saunders, J. (1969) J. Biol. Chem. 244, 3691–3697.
[11] Scocca, J.J. (1971) J. Biol. Chem. 246, 6606–6610.
[12] Odonovan, G.A. and Neuhard, J. (1970) Bacteriol. Rev. 34, 278–343.
[13] Beck, W.S. and Levin, M. (1962) Biochem. Biophys. Acta 55, 245–247.
[14] Kalckar, H.M., Macnutt, W.S., Hoffee, E. and Jorgensen, E. (1952) Biochem. J. 50, 397–400.
[15] Sawula, R.V. and Zamenhoff, P.J. (1974) J. Bacteriol. 117, 1358–1360.
[16] Sawula, R.V., Zamenhoff, S. and Zamenhoff, P.J. (1975) Can. J. Microbiol. 21, 501–509.
[17] Avraham, Y., Yashphe, J. and Grossowicz, N. (1983) Proceedings of the first joint meeting of the Israel Societies of Life Sciences.
[18] Lowry, O.H., Rosenbrough, N.J. and Randall, R.J. (1961) J. Biol. Chem. 193, 265–275.
[19] Vita, A. and Magni, G. (1983) Anal. Biochem. 133, 153–156.

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An *Escherichia coli* enterobactin cluster gene with sequence homology to *trpE* and *pabB*

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Key words: Enterobactin; Chorismate; Isochorismate synthase; Iron regulation

1. SUMMARY

An *Escherichia coli* gene located in the enterobactin gene cluster between *fepB* and *entE* was sequenced. The nucleotide sequence showed a 53% similarity to those of the related genes *pabB* and *trpE*; at the amino acid level, the homology was approximately 20%. The *pabB* and *trpE* genes encode enzymes that use chorismate as a substrate suggesting that the gene sequenced here is *entC*, whose cognate protein is isochorismate synthase. A binding site for the iron regulatory protein Fur was found upstream from *entC* and only 9 bp separated *entC* from *entE*, indicating that *entCE* are cotranscribed.

2. INTRODUCTION

A high affinity iron transport system is induced when *Escherichia coli* is grown in an iron-deficient environment [1]. An iron chelator termed enterobactin (Ent) is synthesized from chorismate via a complex pathway and released into the medium where it binds FeIII. The ferriEnt complex is then transported into the cytoplasm, where iron is released from the chelator for use in cell metabolism. The genes specific for this system, map in a cluster centered at approximately 13.5′ on the *E. coli* map; genes required for Ent biosynthesis have the symbol *ent* and those necessary for ferriEnt transport are designated *fep*.

In a study of transport genes and their products, Pierce and Earhart [2] detected a polypeptide of M_r 29,000 encoded by a gene located between *fepB* and *entE*, a region not previously known to contain a gene. It was subsequently demonstrated [3] that this polypeptide is a truncated form of a 44 kDa whose structural gene was termed *fepF*. The most recently published order for genes in the right half of the Ent gene cluster is *fepB fepF entEBG(CA)* [3,4].

Here we present the nucleotide sequence of the gene for the 44 kDa. Strong homology was detected between it and the genes *pabB* and *trpE*. The proteins encoded by the latter two genes are enzymes that use chorismate as a substrate; the 44 kDa may thus be involved in the first step in Ent biosynthesis, a role carried out by the *entC* product (isochorismate synthase). For this reason and others detailed later as well as for convenience, the structural gene for the M_r 44000 protein will here be termed *entC*.

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Abbreviations: Ent = enterobactin, bp = base pairs.

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3. MATERIALS AND METHODS

3.1. Plasmids

Plasmids pCP111(2), pJP251(2), pMS112(5) and pCP410(6) were used as sources of Ent cluster DNA.

3.2. Materials

Enzymes were obtained either from Bethesda Research Laboratories or Promega Biotech. [α-^{35}S]-dATP was from New England Nuclear Corporation.

3.3. Genetic techniques

M13 replicative form and template DNA were obtained as described by the Bethesda Research Laboratory sequencing manual using *E. coli* strains UT481 (provided by Ian Molineux) and JM101. Enzymatic digestions and ligations of DNA were according to the recommendations of the suppliers. Plasmid DNA was isolated [7] and transformation and transfection of cells [8] was done by previously described procedures.

3.4. DNA cloning and sequencing

A clone (pME2541) encoding the complete *entC* gene was constructed by subcloning the 6.6 kb *Eco*RI fragment of pCP410 into the *Eco*RI site of pJP251. The 4 kb *Eco*RV fragment of pCP111 was subcloned with the *Eco*RV site of pACYC184 to generate pME200. The subclone pJS100 was made by ligating the 2.7 kb *Eco*Ri-*Eco*RV fragment from pCP410 into pGEMBlue cut with *Eco*RI-*Sma*I. Deletion subclones of pME200 extending towards *fepB* and of pJS100 extending counterclockwise into *entC* were prepared with exonuclease III, using the Henikoff method [9] and the Promega Erase-a-Base system, respectively. Restriction fragments for sequencing were subcloned from pME200, pCP111, pMS112 and deletion derivatives of pME200 and pJS100 into the vectors M13mp18 and mp19 [10]. Nucleotide sequences were determined by the dideoxy chain terminating technique [11] using [α-^{35}S]-dATP as described in the Bethesda Research Laboratories sequencing kit. Some pGEMBlue derivatives were sequenced directly by the RNA transcript method described by Promega Biotech. The IBM DNASTAR program was used to analyze sequence data.

4. RESULTS AND DISCUSSION

Fig. 1 shows the nucleotide sequence of *entC*, the 94 bp region upstream from *entC*, the *entC-entE* intergenic region and the information for the first eight amino acids of EntE. Both strands were sequenced in overlapping fashion. The DNA sequence immediately upstream from that shown in Fig. 1 includes regulatory and promoter regions for *fepB* (Elkins, unpublished results.) The direction of transcription for both *ent* genes is clockwise. There are two possible ATG translational initiation codons (positions 94 and 130) for *entC*. We favor the first ATG as the more likely start site because it has an appropriately positioned ribosome binding site (5 bp upstream) and initiates synthesis of a polypeptide with an M_r (42 963) close to 44 000. (The closest Shine-Dalgarno sequence for the 130 start site is 11 bp to its 5′ side.) A binding site for the repressor protein (Fur) for iron-regulated genes is located upstream from *entC* (Fig. 1). The consensus sequence for the Fur binding site is a 19 bp palindrome [12]; the Fur sequence here differs in 4 bp from the consensus sequence and has complete correspondence with the most conserved 5 bp pairs (AATGA-TCATT) [13]. The data do not permit identification of the *entC* promoter as several possible −35 and −10 regions are present upstream. However, the Fur binding site is frequently found in the −35 to −10 region; if this proves to be true for *entC*, the *entC* message would have a leader sequence of approximately 15 nucleotides.

Nine bp separate *entC* and *entE*. A possible Shine-Dalgarno sequence for *entE* is centered 7 bp upstream from the *entE* ATG initiation codon; the 3′A of the *entC* termination codon is the first nucleotide in this site. We suggest that, given the small, promoterless intergenic space between *entC* and *entE*, the fact that both genes are transcribed in the same direction, that both are regulated by iron and the only Fur binding site is upstream from *entC*, and despite the fact that active subclones containing only *entE* have been obtained [4,6], *entCE* normally are transcribed as one unit.

The *entC* nucleotide sequence exhibits extensive homology with *trpE* and *pabB* and there is a

```
    HC
GCGTTGACATAGTGCGCGTTTGCTTTTAGGTTAGCGACCGAAAATATAAATGATAATCATTATTAAAGCCTTTAT
                    100
CATTTTGTGGAGGATGATATGGATACGTCACTGGCTGAGGAAGTACAGCAGACCATGGCAACACTTGCGCCCAAT
                M D T S L A E E V Q Q T M A T L A P N
                                  200
CGCTTTTTCTTTATGTCGCCGTACCGCAGTTTTACGACGTCAGGATGTTTCGCCCGCTTCGATGAACCGGCTGTG
R F F F M S P Y R S F T T S G C F A R F D E P A V
                                                  300
AACGGGGATTCGCCCGACAGTCCCTTCCAGCAAAAACTCGCCGCGCTGTTTGCCGATGCCAAAGCGCAGGGCATC
N G D S P D S P F Q Q K L A A L F A D A K A Q G I

AAAAATCCGGTGATGGTCGGGGCGATTCCCTTCGATCCACGTCAGCCTTCGTCGCTGTATATTCCTGAATCCTGG
K N P V M V G A I P F D P R Q P S S L Y I P E S W
                400 HIII
CAGTCGTTCTCCCGTCAGGAAAAACAAGCTTCCGCACGCCGTTTCACCCGCAGCCAGTCGCTGAATGTGGTGGAA
Q S F S R Q E K Q A S A R R F T R S Q S L N V V E
                                  500
CGCCAGGCAATTCCGGAGCAAACCACGTTTGAACAGATGGTTGCCCGCGCCGCCGCACTTACCGCCACGCCGCAG
R Q A I P E Q T T F E Q M V A R A A A L T A T P Q
S/AC/HC                                           600
GTCGACAAAGTGGTGTTGTCACGGTTGATTGATATCACCACTGACGCCGCCATTGATAGTGGCGTATTGCTGGAA
V D K V V L S R L I D I T T D A A I D S G V L L E

CGGTTGATTGCGCAAAACCCGGTTAGTTACAACTTCCATGTTCCGCTGGCTGATGGTGGCGTCCTGCTGGGGGCC
R L I A Q N P V S Y N F H V P L A D G G V L L G A
                700
AGCCCGGAACTGCTGCTACGTAAAGACGGCGAGCGTTTTAGCTCCATTCCGTTAGCCGGTTCCGCGCGTCGTCAG
S P E L L L R K D G E R F S S I P L A G S A R R Q
          PI N                   800
CCGGATGAAGTGCTCGATCGCGAAGCAGGTAATCGTCTGCTGGCGTCAGAAAAAGATCGCCATGAACATGAACTG
P D E V L D R E A G N R L L A S E K D R H E H E L
                                                PII 900
GTGACTCAGGCGATGAAAGAGGTACTGCGCGAACGCAGTAGTGAGTTACACGTTCCTTCTTCTCCACAGCTGATC
V T Q A M K E V L R E R S S E L H V P S S P Q L I
                                RI
ACCACGCCGACGCTGTGGCATCTCGCAACTCCCTTTGAAGGTAAAGCGAATTCGCAAGAAAACGCACTGACTCTG
T T P T L W H L A T P F E G K A N S Q E N A L T L
                1000
GCCTGTCTGCTGCATCCGACCCCCGCGCTGACGGCTTTCCCGCATCAGGCCGCGACCCAGGTTATTGCTGAACTG
A C L L H P T P A L T A F P H Q A A T Q V I A E L
                                  1100
GAACCGTTCGACCGCGAACTGTTTGGCGGCATTGTGGGTTGGTGTGACAGCGAAGGTAACGGCGAATGGGTGGTG
E P F D R E L F G G I V G W C D S E G N G E W V V
                                                  1200
ACCATCCGCTGCGCGAAGCTGCGGGAAAATCAGGTGCGTCTGTTTGCCGGAGCGGGGATTGTGCCTGCGTCGTCA
T I R C A K L R E N Q V R L F A G A G I V P A S S

CCGTTGGGTGAGTGGCGCGAAACAGGCGTCAAACTTTCTACCATGTTGAACGTTTTTGGATTGCATTAAGGAGCG
P L G E W R E T G V K L S T M L N V F G L H .
                1302
AGGATGAGCATTCCATTCACCCGCTGG
   M S I P F T R W
```

Fig. 1. Nucleotide sequence of the *entC* gene with its predicted amino acid sequence shown in one-letter code below the DNA sequence. The non-coding strand is shown, the Fur binding site is boxed, possible Shine-Dalgarno sequences are underlined, and restriction sites are indicated by the names above their recognition sequence as follows: A, *Ava*I; Ac, *Acc*I; HIII, *Hin*dIII; Hc, *Hin*cII; N, *Nru*I; PI, *Pvu*I; PII, *Pvu*II; RI, *Eco*RI; RV, *Eco*RV; S, *Sal*I.

```
EntC
PabB
TrpE  M Q T Q K P T L E L L T C E G A Y R D N P T A L F H Q L C G D R P A T L L

                                                  M K T L S P A V I T L L W R Q D A
L E S A D I D S K D D L K S L L L V D S A L R I T A L G D T V T I Q A L S G N G

A E F Y F S R L S H L P W A M L L H S G Y A D H P Y S R F D I V V A E P I C T L
E A L L A L L D N A L P A G V E S E Q S P N C R V L R F P P V S P L L D E D A R

            M D T S L A E E V Q Q T M A T L A P N R F F F M S P Y R S F T T S G
T T F G K E T V V S E S E K R T T T T D D P L Q V L Q Q V L D R A D I R P T H N
L C S L S V F D A F R L L Q N L L N V P K E E R E A M F F S G L F - S Y D L V A

C F A R F D E P A V N G D S P D S P F Q Q K L A A L F A D A K A Q G I K N P V M
E D L P F Q G G A L G L F G Y D L G R R F E S L P E I A E Q D I V L P D M A V G
G F E D L P Q L S A E N N C P D F C F - - Y L A E T L M - - V I D H Q K K S T R

V G A I P F D P R Q P S S L Y I P E S W Q S F S R Q E K Q A S A R R F T R S Q S
I Y D W A L I V D H Q R H T V S L L S H N D V N A R R A W L E S Q Q F S P Q E D
I Q A S L F A P N E E E K Q R L T A R L N E L - R Q Q L T E A A P P L P V V - S

L N V V E R Q - A I P E Q T T F E Q M V A R A A A L T A T P Q V D K V V L S R L
F T L T S D W Q S N M T R E Q Y G E K F R Q V Q E Y L H S G D C Y Q V N L A Q R
V P H M R C E C - N Q S D E E F G G V V R L L Q K A I R A G E I F Q V V P S R R

I D I T T D A A I D S G V L L E R L I A Q N P V S Y N F H V P L A D G G V L L G
F H - A T Y S G D E W Q A F L Q - L N Q A N R A P F S A F L R L E Q G A I - L S
F S L P C P S P L A A Y Y V L K K - - - S N P S P Y M F F M Q - D N D F T L F G

A S P E L L L R K D G - E R - F S S I P L A G S A - R - R Q P D E V L D R E A G
L S P E R F I L C D N - S - E I Q T R P I K G T L P - - R L P D P Q E D S K Q A
A S P E S S L K Y D A T S R Q I E I Y P I A G T R P R G R R A D G S L D R D L D

N R - - L - L A - S E K D R H E H - E L V T Q A M K E V L R E - R S S E L H V P
V K - - L - - A N S A K D R A E N L M I V D L M R N D I G R V A V A G S V K V P
S R I E L E M R T D H K E L S E H L M L V D L A R N D L A R I C T P G S R Y V A

S S P Q L I T T P T L W H L A T P F E G K A N S Q E N A L T L A C L L H P T P A
E L F V V E P F P A V H H L V S T I T A Q L P E Q L H A S D L L R A A F P G G S
D L T K V D R Y S Y V M H L V S R V V G E L R H D L D A L H A Y R A C M N M G T

L T A F P H Q A A T Q V I A E L E P F D R E L F G G I V G W C D S E G N G E W V
I T G A P K V R A M E I I D E L E P Q R R N A W C G S I G Y L S F C G N M D T S
L S G A P K V R A M Q L I A E A E G R R R G S Y G G A V G Y F T A H G D L D T C

V T I R C A K L R E N Q V R L F A G A G I V P A S S P L G E W R E T G V K L S T
I T I R T L T A I N G Q I F C S A G G G I V A D S Q E E A E Y Q E T F D K V N R
I V I R S A L V E N G I A T V Q A G A G V V L D S V P Q S E A D E T R N K A R A

M L N V F G L H
I L K Q L E K
V L R A I A T A H H A Q E T F
```

Fig. 2. Amino acid alignment of the products on the *entC, pabB* and *trpE* genes. Loci with two or more identical amino acids are underlined.

correspondingly high degree of homology among the proteins (Fig. 2); homologous regions are particularly obvious in the carboxy termini of the proteins. (Goncharoff and Nichols [14] have noted the relatedness between *pabB* and *trpE* at the amino acid and nucleotide level.) The *trpE* and *pabB* genes both show similarities of 53% with *entC*. In the common portions of the three amino acid sequences (Fig. 2), the homologies are 19%, 23.9% and 22.3% for EntC-PabB, PabB-TrpE, and EntC-TrpE, respectively. The *trpE* and *pabB* products both (i) bind chorismic acid [14,15] and (ii) are components of enzymes (anthranilate synthase and *p*-aminobenzoate synthase, respectively) composed of two non-identical subunits. The product of the gene sequenced here may thus be part of a two component enzyme that utilizes chorismate as a substrate. The first specific step in Ent biosynthesis involves the conversion of chorismate to isochorismate, a reaction catalyzed by isochorismate synthase, the *entC* product. In what immediately follows we argue that the sequence reported here is for *entC* and attempt to reconcile this conclusion with prior work which indicates that *entC* maps near *entA*, not *entE*, and that the products, complete and truncated, of the gene sequenced here are associated with membrane [2,3], unlike isochorismate synthase.

Previous studies on *entC* and its product have yielded confusing results, possibly because only one mutant allele of *entC (entC401)* [16] exists. The *entC401* mutation maps close to *entA* [4,17]. Of the reports [5,18,19] indicating that complementing activity for this *entC* allele can be separated from that for *entA*, only one has not been challenged [4]. Recent efforts to distinguish *entA* from *entC* have been unsuccessful, as have been efforts to resolve their products from one another ([4,6] Staab and Earhart, unpublished observations). Also, the isochorismate synthase of cells harboring an *entC401* mutation has not been measured directly [16,18] and in fact studies on menaquinone biosynthesis indicate such cells have a significant amount of synthase activity [20]. If one postulates that the *entC* and *entA* polypeptides interact, *entC401* may actually be an unusual allele of *entA* resulting in an enzymatically active *entA* protein but one that is unable to associate with and activate the *entC* polypeptide. The *entA* protein would then formally correspond to the second components of *p*-aminobenzoate synthase and anthranilate synthase [21] although it differs from the products of the *pabA* and *trp(G)D* genes in that it is not a glutamine amidotransferase. We and McIntosh (personal communication) now regard our previous localization results [2,3] as inconclusive. Additional localization studies (Elkins, unpublished results) with plasmid pME2541 resulted in detection of EntC, EntE, EntB and EntA all in both membrane and soluble fractions, presumably because these genes are present on a multicopy plasmid [22]. In view of the sequence homology results and the absence of firm evidence to the contrary, we suggest that the gene located between *fepB* and *entE* is *entC*.

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REFERENCES

[1] Earhart, C.F. (1987) in: Iron Transport in Microbes, Plants and Animals (Winkelmann, G., van der Helm, D. and Neilands, J.B. eds.), pp. 67–84, VCH Verlagsgesellschaft, Weinheim.
[2] Pierce, J.R. and Earhart, C.F. (1986) J. Bacteriol. 166, 930–936.
[3] Ozenberger, B.A., Nahlik, M.S. and McIntosh, M.A. (1987) J. Bacteriol. 169, 3638–3646.
[4] Nahlik, M.S., Fleming, T.P. and McIntosh, M.A. (1987) J. Bacteriol. 169, 4163–4170.
[5] Laird, A.J., Ribbons, D.W., Woodrow, G.C. and Young, I.G. (1980) Gene 11 (Amst.), 347–357.
[6] Pickett, C.L., Hayes, L. and Earhart, C.F. (1984) FEMS Microbiol. Lett 24, 77–80.
[7] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523.
[8] Cohen, S.N., Chang, A.C.Y. and Hsu, L. (1972) Proc. Natl. Acad. Sci. USA 69, 2110–2114.
[9] Henikoff, S. (1984) Gene (Amst.) 28, 351–359.

[10] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene (Amst.) 33, 103–119.
[11] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[12] de Lorenzo, V., Wee, S., Herrero, M. and Neilands, J.B. (1987) J. Bacteriol. 169, 2624–2630.
[13] Calderwood, S.B. and Mekalanos, J.J. (1988) J. Bacteriol. 170, 1015–1017.
[14] Goncharoff, P. and Nichols, B.P. (1984) J. Bacteriol. 159, 57–62.
[15] Ito, J., Cox, E.C. and Yanofsky, C. (1968) J. Bacteriol. 97, 725–733.
[16] Young, I.G., Langman, L., Luke, R.K.J. and Gibson, F. (1971) J. Bacteriol. 106, 51–57.
[17] Laird, A.J. and Young, I.G. (1980) Gene 11 (Amst.), 359–366.
[18] Woodrow, G.C., Young, I.G. and Gibson F. (1975) J. Bacteriol. 124, 1–6.
[19] Fleming, T.P., Nahlik, M.S. and McIntosh, M.A. (1983) J. Bacteriol. 156, 1171–1177.
[20] Bentley, R. and Meganathan, R. (1987) in *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology (Neidhardt, F.C., ed.), vol. 1, pp. 512–520, American Society for Microbiology, Washington, D.C.
[21] Kaplan, J.B. and Nichols, B.P. (1983) J. Mol. Biol. 168, 451–468.
[22] Coderre P.E. and Earhart, C.F. (1984) FEMS Microbiol. Lett. 25, 111–116.

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Effects of penicillin on the synthesis of membrane proteins of *Chlamydia trachomatis* LGV2 serotype

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Key words: Penicillin; Outer membrane proteins; *Chlamydia*; Protein expression

1. SUMMARY

Cultures of LLC-MK2 cells were infected with *Chlamydia trachomatis* LGV2 serotype and incubated in medium containing 200 units per ml of penicillin. Forty-eight hours after infection, cells were removed and chlamydiae purified. By Coomassie blue-stained polyacrylamide gels, penicillin-treated chlamydiae showed to be deficient in 60 K and 62 K outer membrane proteins. By using a more sensitive immunoblotting technique, in conjunction with human and animal immune sera containing antibodies specific for the 60 K and 62 K proteins, the synthesis of the 60 K protein turned out to be completely inhibited by penicillin, whereas the 62 K protein seemed to be unaffected. The synthesis of the major outer membrane protein (MOMP), studied with a specific monoclonal antibody by using immunofluorescence and immunoblotting techniques, did not seem affected by penicillin. These data indicate that penicillin interferes with conversion of reticulate body to elementary body of *C. trachomatis* LGV2 serotype, by affecting the synthesis of the 60 K cysteine-rich outer membrane protein.

2. INTRODUCTION

Chlamydiae are obligate intracellular parasites which present a unique developmental cycle [1], characterised by the alternation of an infectious elementary body (EB) with a non-infectious reticulate body (RB) which multiplies by binary fission for about 21–24 h. At that time the RB reorganizes into EB. Both developmental forms have an inner and outer membrane [2]. The outer membrane proteins of EBs are extensively cross-linked by disulfide bonds, whereas those of RBs are not [3–5]. The outer membrane of EBs presents the cysteine-rich proteins of 60 K and 62 K, whereas the outer membrane of RBs is deficient in these polypeptides [6,7].

The space between the outer and inner membrane of chlamydiae does not seem to be occupied by a structure analogous to peptidoglycan, and muramic acid is apparently undetectable in preparations of either RBs and EBs [8,9]. Interestingly, many in vitro studies have shown that chlamydial RB cells stop dividing and do not convert to EBs in the presence of very low con-

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centrations of penicillin [10–12]. The possible targets for penicillin in *C. trachomatis* are three penicillin-binding proteins (PBPs) which were identified by Barbour et al. [8] in the Sarkosyl-soluble fractions of both EBs and RBs. However, the mechanism of this action was not elucidated. In the present study we report that penicillin inhibited the synthesis of the 60 K membrane protein of *C. trachomatis* LGV2 serotype.

3. MATERIAL AND METHODS

3.1. Growth and purification of chlamydiae

A *C. trachomatis* LGV2-serotype strain was used. Chlamydiae were grown in monolayer of LLC-MK2 cells (a continuous cell line prepared from Rhesus monkey kidney) in Eagle essential medium supplemented with 10% (v/v) fetal calf serum, in the presence of cycloheximide (2 μg/ml). The RBs and EBs collected 21 and 48 h after infection, respectively, were purified by centrifugation in discontinuous Renografin (E.R. Squibb and Sons, Princeton, NJ) gradients, as described [13].

To obtain penicillin-treated chlamydiae, penicillin G sodium (Carlo Erba, Milan, Italy) was added (200 units/ml) to the culture medium immediately after infection. At 48 h cell monolayers were harvested and chlamydiae purified using 30 : 35 : 40% Renografin gradients. Chlamydial forms were obtained at the interface of 35–40% discontinuous gradients [14].

3.2. Penicillin susceptibility

Two-day old LLC-MK2 cell monolayers seeded in tube were inoculated with 1 ml of *C. trachomatis* LGV2 to yield 10^3 to 10^4 inclusion-forming units per ml, in the presence of 200 units/ml of penicillin. It was shown by Tamura and Manire [12] that penicillin at concentration $\geqslant$ 50 units/ml inhibited the infectivity of chlamydiae by more than 98%. At various time intervals after infection, the cells were fixed with methanol and tested both with Giemsa stain and with immunofluorescence (IF), using the monoclonal B1-10 which showed anti-MOMP reactivity [15] Bound antibodies were detected with fluorescein-conjugated anti-mouse immunoglobulins (Dakopatts Copenhagen, Denmark).

In order to evaluate the effect of penicillin (200 units/ml) on infectivity of chlamydiae-infected cells, the monolayers incubated in the presence of penicillin for 48 h were assayed for infectivity (i.e., inclusion-forming units) by passage in antibiotic-free monolayers. Cells were scraped off the cover slip, and inoculated into fresh LLC-MK2 cell monolayers. After incubation for 48 h, the cultures were fixed as above and stained both by Giemsa and IF.

3.3. SDS-PAGE

The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli [16] was followed, using 12.5% (w/v) acrylamide concentration in the separating gel and gel dimensions of $15 \times 12 \times 0.15$ cm. Electrophoresis (20 mA, constant current) was continued until dye front had migrated 10 cm onto the separating gel.

3.4. Immunological methods

The serum from a female patient with *C. trachomatis* isolation-positive cervicitis, with specific antibodies against several chlamydial proteins including 60 K, 62 K and MOMP, as detected by immunoblotting [14], was used. A polyclonal guinea-pig serum raised against purified EBs of *C. trachomatis* LGV2 serotype, was also used. Immunoblotting was performed by a standard protocol [17]. After transfer, the nitrocellulose paper was incubated for 30 min at room temperature in 50 mM sodium phosphate, 0.15 M NaCl, 0.05% (v/v) Tween-20 (pH 7.3), 0.02% NaN_3. The nitrocellulose paper was then cut into strips, which were incubated with human and guinea-pig immune sera or with monoclonal antibody B1-10 for 16 h at room temperature, under gentle agitation. The strips were washed 3 times by PBS, followed by incubation for 2 h at room temperature with peroxidase-labelled rabbit immunoglobulins to mouse, human and guinea pig IgG (Dakopatts), respectively. The nitrocellulose strips were then washed three times for 10 min with PBS and transferred to distilled water. The

immunoblots were developed with a solution of 40 mg benzidine (Fluka, Buchs, Switzerland) dissolved in 4 ml acetone, 96 ml PBS and 0.1 ml hydrogen peroxide from 33% stock solution. After development, the blots were repeatedly rinsed with distilled water. IF staining of LLC-MK2 cells infected with chlamydiae was performed by standard methods with monoclonal antibody B1-10 and fluorescein-conjugated second antibodies [15].

4. RESULTS

In a first series of experiments, cell cultures infected by *C. trachomatis* and treated with penicillin immediately after infection, were tested by indirect immunofluorescence staining with monoclonal antibody B1-10 which reacts positively with the MOMP of LGV2 in the immunoblotting technique [15]. By 12–14 h postinfection, a positive reaction was observed either in cell culture treated or untreated by penicillin. The intensity of fluorescence and the dimension of inclusions detected both in penicillin-treated and untreated cultures peaked at 48 h after infection. At that time, large, fluorescent ring-like structures were detectable inside the vacuoles of infected cells treated by penicillin (Fig. 1). However, the infectivity of these preparations was reduced by more than 98% in comparison with controls (data not shown) [12].

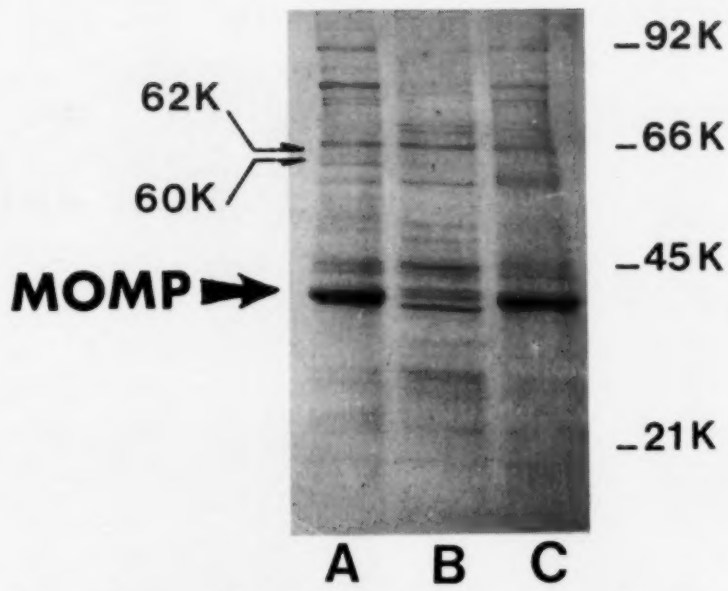

Fig. 2. Coomassie blue-stained polyacrylamide gels of *C. trachomatis* LGV2 grown in LLC-MK2 cells. RBs (C) and EBs (A) were harvested and purified 24 and 48 h after infection, respectively. *C. trachomatis* infected cells were treated immediately after infection with penicillin (200 units/ml) and the abnormal RB cells were purified 48 h after infection (B).

Chlamydiae grown for 48 h in the presence of penicillin were purified by Renografin gradients and proteins were separated by SDS-PAGE. Fig. 2 shows the SDS-PAGE profile of penicillin-treated

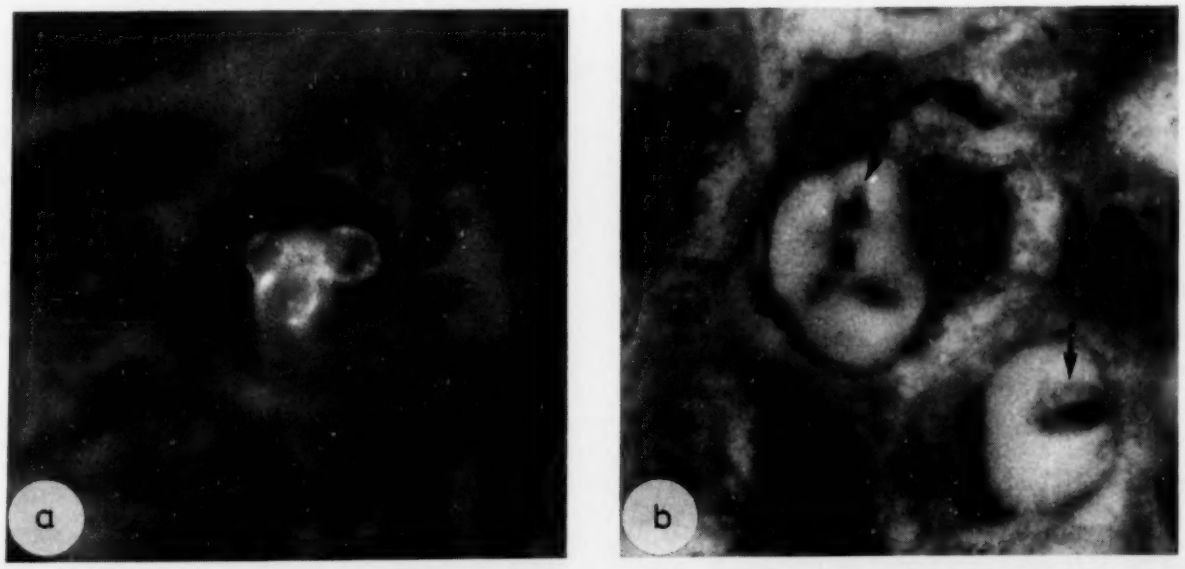

Fig. 1. Photomicrographs of preparations of LLC-MK2 cells infected with *C. trachomatis* LGV2 serotype treated with penicillin (200 units/ml) for 48 h. Large rings of fluorescence (abnormal RBs) were observed when preparations were tested by IF technique using a monoclonal antibody reactive with the MOMP of *C. trachomatis* (a). Vacuoles containing abnormally large RBs (arrows) were observed in Giemsa stained preparations (b). (×2500)

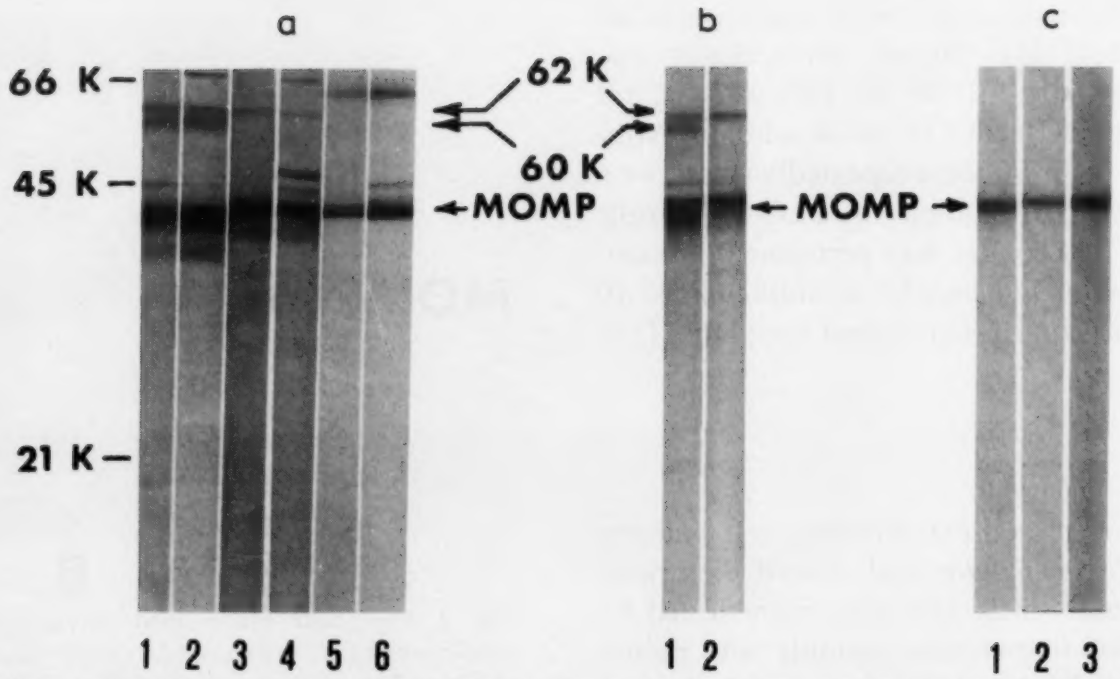

Fig. 3. Immunoblotting of purified and unpurified preparations of *C. trachomatis* treated or untreated with penicillin, with human and guinea-pig immune sera with specific antibodies against several chlamydial proteins including the 60 K, 62 K and the MOMP. The purified chlamydial preparations were also tested with a monoclonal antibody reactive with the MOMP. Frame a: Lanes 1, 3, 5 represent immunoblots of human serum with purified EBs, with penicillin-treated and purified chlamydiae, and with RBs respectively. Lanes 2, 4, 6 as lanes 1, 3, 5 but guinea-pig immune serum was used. Frame b. Immunoblots of human serum with crude extract of *C. trachomatis* infected cells treated (lane 2) or untreated (lane 1) with penicillin for 48 h. Frame c. Immunoblots of the MOMP-reactive monoclonal antibody B1-10 tested with purified EBs (lane 1), RBs (lane 2) and with penicillin-treated and purified chlamydiae (lane 3).

chlamydiae in comparison with untreated *C. trachomatis* RBs and EBs purified 21 and 48 h after infection, respectively. Many proteins were revealed in the purified preparations, the most prominent being the MOMP. The 60 K and 62 K outer membrane proteins were present in EB preparations and absent in RB preparations. The 60 K and 62 K proteins were also undetectable in penicillin-treated chlamydiae, examined 48 (or 72) h after infection.

The immunoblot analysis of penicillin-treated chlamydiae in comparison with untreated preparations by using human and animal immune sera with specific antibodies against the 60 K and 62 K proteins and the MOMP-reactive B1-10 monoclonal antibody, is reported in Fig. 3. Both human and guinea-pig immune sera recognized several proteins, among others the 60 K and 62 K proteins, in EB preparations. As expected, no reaction was observed in RB preparations in the 60 K and 62 K positions. In penicillin-treated chlamydiae, the 60 K protein was not detected, whereas a clear reaction was observed in the 62 K position (Fig. 3a). A similar pattern of reaction was observed in unpurified chlamydiae (Fig. 3b). The MOMP was detected by monoclonal antibody in all three preparations (Fig. 3c).

5. DISCUSSION

Chlamydial infections are not generally treated with beta-lactam antibiotics. However, in vitro studies showed that chlamydiae, which have not detectable muramic acid, are susceptible to penicillin [10–12]. In the presence of very low concentration of penicillin, *C. trachomatis* RB cells stop dividing and do not convert to EBs, whereas any penicillin which bounds to EBs does not affect infectivity or transition to RBs [8]. Electron microscopy studies of Matsumoto and Manire [11] have shown that in the presence of penicillin, in the vacuoles of treated cell culture, large RBs are present, in which both the outer cell envelope and the cytoplasmic membrane are preserved. The observations of Stirling et al. [18] based on metabolic

studies, supported the view that the formation of cysteine-containing structures is essential in RB-EB transformation, since conversion of RBs to EBs is severely retarded in the absence of cysteine in the growth medium. Further studies by Hatch et al. [5] showed that EBs but not RBs harvested 21 h postinfection, contained extraordinarily cysteine-rich outer membrane proteins of 60 K and 62 K. The time and extent of synthesis of cysteine-rich proteins seems to be regulated by processes that coincide in time with transformation of RBs to EBs [7].

The data of the present study showed that penicillin prevents the synthesis of the 60 K cysteine-rich protein in chlamydial developing forms. Since the cysteine-rich proteins seem to be essential for the transformation of RBs into EBs [5,18], penicillin may inhibit chlamydial growth by affecting the synthesis of the 60 K protein. The mechanisms of this action are not known, however.

Barbour et al. [8] demonstrated the presence of three PBS associated with cytoplasmic membrane or cytoplasm of *C. trachomatis,* suggesting that penicillin is acting on a target not necessarily involved in transpeptidation reactions. It is also known that some of the PBPs associated with the inner membrane of *Escherichia coli* have been identified either with penicillin-sensitive enzymes involved in peptidoglycan synthesis or, when enzyme specificity remains undefined, with a process such as cell septation or cylindrical growth [19,20].

In a recent report, Newhall [7] showed that the 60 K and 62 K proteins of *C. trachomatis* LGV2 serotype were extensively cross-linked during most of the growth cycle. Newhall suggested that these data may indicate an intracellular, possibly enzymatic, cross-linking mechanism, present in addition to an auto-oxidation mechanism that occurs upon host cell lysis and exposure to extracellular environment [6]. An extension of this suggestion is that one or more of the chlamydial PBPs is involved in the enzymatic activity which possibly mediates the intracellular cross-linking process of the 60 K cysteine-rich protein of *C. trachomatis* RB cells.

REFERENCES

[1] Schachter, J. and Caldwell, H.D. (1980) Annu. Rev. Microbiol. 34, 285–309.
[2] Newhall, W.J. and Jones, R.B. (1983) J. Bacteriol. 154, 998–1001.
[3] Bavoil, P., Ohlin, A. and Schachter, J. (1984) Infect. Immun. 44, 479–485.
[4] Hackstadt, T., Todd, W.J. and Caldwell, H.D. (1985) J. Bacteriol. 161, 25–31.
[5] Hatch, T.P., Allan, I. and Pearce, J.H. (1984) J. Bacteriol. 157, 13–20.
[6] Hatch, T.P., Miceli, M. and Sublett, J.E. (1986) J. Bacteriol. 165, 379–385.
[7] Newhall, W.J. (1987) Infect. Immun. 55, 162–168.
[8] Barbour, A.G., Amano, K., Hackstadt, T., Perry, L. and Caldwell, H.D. (1982) J. Bacteriol. 151, 420–428.
[9] Garret, A.J., Harrison, M.J. and Manire, G.P. (1974) J. Gen. Microbiol. 80, 315–318.
[10] Kramer, M.J. and Gordon, F.B. (1971) Infect. Immun. 3, 333–341.
[11] Matsumoto, A. and Manire, G.P. (1970) J. Bacteriol. 104, 1332–1337.
[12] Tamura, A. and Manire, G.P. (1968) J. Bacteriol. 96, 875–880.
[13] Cevenini, R., Sarov, I., Rumpianesi, F., Donati, M. and Melega, C. (1984) J. Clin. Pathol. 37, 686–691.
[14] Cevenini, R., Rumpianesi, F., Donati, M., Moroni, A., Sambri, V. and La Placa, M. (1986) J. Clin. Pathol. 39, 1313–1316.
[15] Cevenini, R., Donati, M., Sambri, V., Rumpianesi, F. and La Placa, M. (1987) FEMS Microbiol. Letters 42, 47–51.
[16] Laemmli, U.K. (1970) Nature (London) 227, 680–685.
[17] Cevenini, R., Rumpianesi, F., Sambri, V. and La Placa, M. (1986) J. Clin. Pathol. 39, 325–327.
[18] Stirling, P., Allan, I. and Pearce, J.H. (1983) FEMS Microbiol. Lett. 19, 133–136.
[19] Spratt, B.G. (1978) Sci. Prog. (London) 65, 101–128.
[20] Suzuki, H., Nishimura, Y. and Hirota, Y. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 664–668.

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Exocellular products from *Bifidobacterium adolescentis* as immunomodifiers in the lymphoproliferative responses of mouse splenocytes

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1. SUMMARY

Extracts of exocellular products from *Bifidobacterium adolescentis* and their fractions were tested as immunomodifiers of the murine lymphoproliferative response. The extracts induced a dose dependent blastogenic activation which was higher for the fractions rich in polysaccharides. When interacting with lectins, neither the extracts nor the protein-rich fractions produced any effect on the lymphoproliferative response. The carbohydrate-rich fractions stimulated DNA synthesis in LPS-sensitive B lymphocytes but decreased it in Con A-sensitive T lymphocytes.

2. INTRODUCTION

Bifidobacterium species are particularly interesting because their interaction with the gastrointestinal mucose produces a concomitant modification of the host resistance to bacterial infections [1–3]. Also, their role as secondary invaders of the gastrointestinal tract in human [4] and avian [5] hosts may be important. Ecological significance of host/bifidobacteria interactions would need an important taxonomic investigation at an immunological level. Studies on transaldolases of the genus *Bifidobacterium* allowed identification to arrange them into distinct strain clusters based on the values of the dissimilarity index of their respective transaldolases [6–8].

In more recent years, there have been several reports of the antitumor activity of Bifidobacteria [reviewed in 9]. Breuillaud [10] considered these bacteria as very interesting for their immunostimulant ability. However, only few studies have focused on identification of the active component(s) responsible for the immunobiological activities of Bifidobacteria [11]. Therefore, we initiated studies to determine whether exocellular extracts from *Bifidobacterium adolescentis* can modify the lymphoproliferative response of murine splenocytes and to identify the bacterial component involved in these processes.

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3. MATERIAL AND METHODS

3.1. Organisms, media, growth conditions and preparation of extracts

Bifidobacterium adolescentis (ATCC 15703) was grown at 37°C in a liquid medium (3.7% w/v) Difco Brain Heart Infusion, and 1% (w/v) Glucose (Merck) in a 2-l fermentor under an anaerobic atmosphere of $CO_2 : N_2$ (10 : 90), inoculated with a 100-ml stationary batch culture. Bacterial culture was harvested after 48 h and centrifuged at 3500 $\times g$. The supernatant was concentrated (1 : 10) in a Büchi 110 Rotavapor, and dialyzed in a 22/32 Visking Tubing (Scient. Inst. Centre, Ltd. London) against deionized water for 24 h. The non-diffusible material was saturated by 80% with $(NH_4)_2SO_4$, the precipitate was collected by centrifugation, resuspended in water and extensively dialyzed against deionized water. The supernatant from the $(NH_4)_2SO_4$ saturation was treated in the same way.

Both fractions were precipitated with 96% (v/v) ethanol and allowed to stand at 4°C for 48 h. After centrifugation, the crude precipitates (E_1 and E_2) were suspended in cold deionized water and lyophilized. Further purification of the precipitates was carried out by gel filtration in a Sepharose 6B column (Pharmacia K26/40) eluted with 0.5 M amonium acetate at a flow rate of 12 ml/h; 4 ml aliquots were collected in a LKB (Bromma, Sweden) fraction collector connected to a LKB Uvicord. Fractions with the same absorbance pattern were pooled, concentrated and lyophilized.

3.2. Chemical analysis

Protein and carbohydrate were determined by the Lowry [12] and the anthrone [13] methods, respectively.

The protein composition of the extracts and of their fractions was elucidated by the sodium dodecylsulphate polyacrylamide gel electrophoresis by the method of Laemli [14]. On each gel, a calibration solution of proteins (Bio-Rad) was included. Silver nitrate stain and Coomassie Blue R-250 were used for staining. The molecular weights of isolated protein bands were calculated by their relative mobilities, according to Weber and Osborn [15].

Neutral sugars were analyzed by gas-liquid chromatography (GLC) on 3% SP-2340 on 100/120 Supercolpost, as the alditol acetates after hydrolysis with 4n H_2SO_4 for 5 h at 100°C under N_2 atmosphere [16,17]. Inositol (Sigma) was the internal standard. Samples were reduced with sodium borohydride. Standards containing arabinose, threose, xylose, glycerol, rhamnose, glucose, galactose, manose, galactosamine and glucosamine (Sigma) were prepared with the internal standard to determine relative molar responses.

3.3. Murine splenocyte culture and blastogenesis assays

Splenocyte cellular cultures from male BALB/c mice (8–10 weeks old) were obtained as described elsewhere [18]. The effect of the bacterial samples on the lymphoproliferative response and their interaction with the mitogens of T (Concavalin A, Con A) and B (lipopolysaccharide; LPS) lymphocytes were studied by measuring the incorporation of ^{3}H-thymidine in sample-treated and untreated control cultures of murine splenocytes [19]. Con A was from Calbiochem and the *E. coli* LPS from Difco. Viability of lymphocytes was also tested by the trypan blue exclusion test [20].

4. RESULTS AND DISCUSSION

The growth of *B. adolescentis* in a fermentor gave the maximum yield at 48 h, 40 h earlier than in the common batch cultures. The extracts E_1 and E_2 obtained from exocellular bacterial material showed a similar absorption pattern at 274 nm when fractionated in Sepharose 6B column (Fig. 1). Three pools were made: A, containing material eluted near the void volume; B, containing material with no absorption at 254-274 nm; and C, containing the highly absorptive material of smaller molecular weight. Both extracts (E_1, E_2) and their corresponding A, B and C fractions reacted in Ouchterlony immunoprecipitation assays with whole-cell serum as antibodies.

The chemical analysis of the crude extracts E_1 and E_2 and of their fractions A, B and C showed

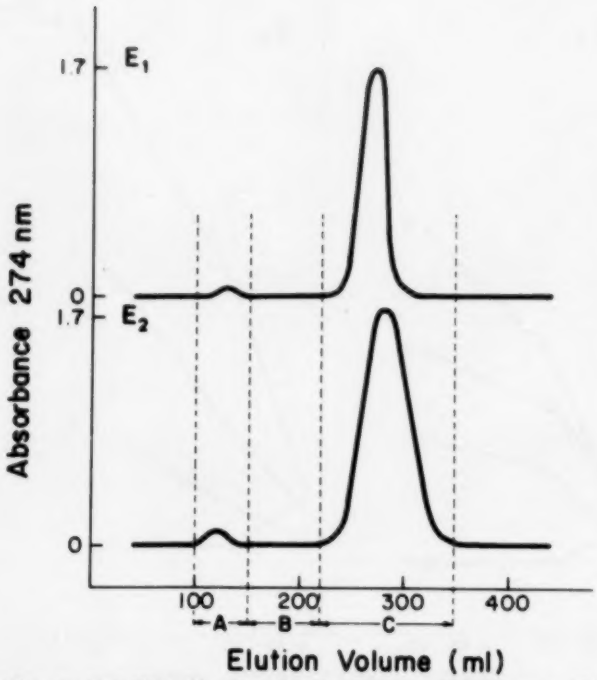

Fig. 1. Gel filtration in the Sepharose 6B column of extracts E_1 and E_2 from cultures of *B. adolescentis.* The fractions A, B and C were pooled as indicated.

that the ratio protein/reducing substances (PR/RS) was different in each fraction (Table 1). As expected, E_1 consisted mainly of proteins which were eluted mostly in fraction C (Fig. 1). The protein patterns of the extracts and the fractions were heterogeneous (Fig. 2). Although E_1 and E_2 crude extracts shared peptides of a similar apparent molecular weight, E_1 appeared more complex. Five peptides apparent in E_1 crude extract were not observed in E_2; these proteins had a molecular weight of 87, 71.2, 51.8 48.0 and 26.3

Table 1

Chemical composition of the extracts and of their fractions.

Sample	Composition (%)		PR/RS [a]
	Protein	Reducing substances	
Extract E_1	44.1	1.5	29.0/1
Fraction A	4.1	2.2	1.9/1
Fraction B	3.0	11.6	0.3/1
Fraction C	49.7	1.1	43.6/1
Extract E_2	69.2	9.0	7.7/1
Fraction A	9.7	9.5	1.0/1
Fraction B	5.2	51.5	0.1/1
Fraction C	48.4	5.1	9.3/1

[a] Protein/Reducing substances.

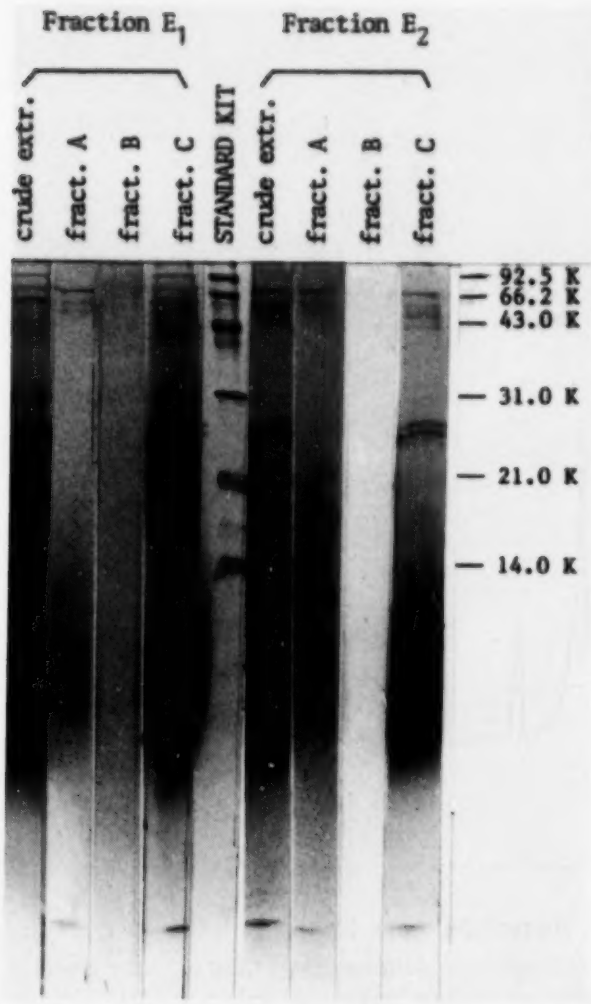

Fig. 2. Sodium dodecylsulphate-polyacrylamide gel electrophoresis of the extracts E_1 and E_2 and their fractions A, B and C from *B. adolescentis.* Gels were loaded with: 120 μg (E_1); 180 μg (E_2, A and C from E_1): 200 μg (A and C from E_2); and 240 μg (both fractions B). Migration distance of the standard proteins (M_r) are indicated on the right.

kDa, respectively. In E_2 a band of 68.8 kDa could be seen. Fractions A, from E_1 and E_2 showed three similar peptides. Fractions B did not display any proteic band. Both fractions C displayed almost every peptide observed in their respective crude extracts. However the attempts to dilucidate the carbohydrate composition by silver staining failed even when gels were loaded with 240 μg of sample.

In GLC analysis of the extracts E_1 and E_2, a similar qualitative composition with only seven monosaccharides could be detected. As an example, in Fig. 3 the E_2 GLC chromatogram is presented. These extracts and their B fractions, whose composition appears in Table 2, had xylose, man-

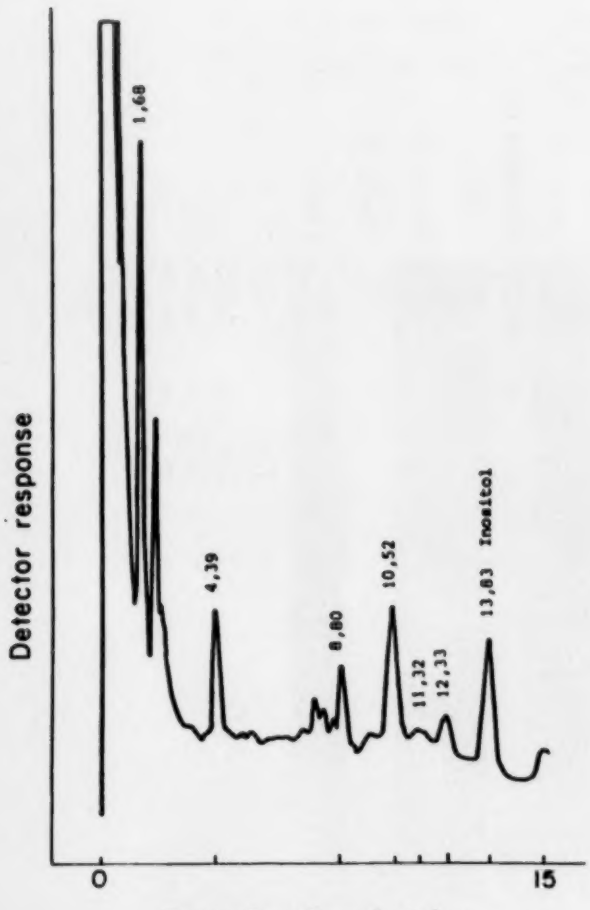

Fig. 3. Chromatogram of alditol acetates from E_2, were glycerol, threose, arabinose, xylose, mannose, galactose and glucose. Numbers indicate retention times in minutes.

nose and arabinose as main neutral sugars. The carbohydrate composition of the active fractions from *B. adolescentis* extracts indicates that no typical sugar of pectidoglycan (glucosamine or muramic acid) were present. The high amount of mannose, xylose and arabinose in both fractions B

Table 2

Neutral sugar composition of the E_1, E_2 extracts and their corresponding B fractions.

Sugar	Carbohydrate content (nmol/mg)			
	E_1	B_1	E_2	B_2
Glucose	6.2	5.8	15.0	9.8
Galactose	1.2	0.97	4.5	4.2
Mannose	27.2	26.4	65.3	59.6
Xylose	47.2	23.2	38.3	25.2
Arabinose	25.4	24.3	12.5	12.3
Threose	11.6	12.7	10.9	11.4
Glycerol	93.0	91.5	98.5	89.3

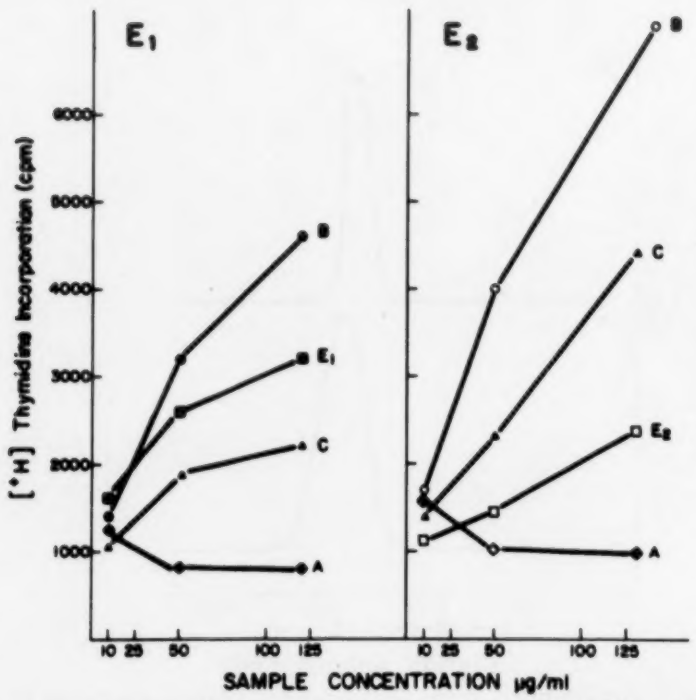

Fig. 4. Murine splenocyte intrinsic mitogenesis produced by the extracts E_1 and E_2 and by their fractions A, B and C. Values are represented as net [^{3}H]-thymidine incorporation. cpm values in PBS control cultures were 1.650 ± 128.

suggest a role for them in the biological activity of fraction B (see below).

After observing that viability of splenic lymphocytes was not affected by the different samples of extracts at doses lower than 200 μg/ml, studies on lymphoproliferative responses were performed. An intrinsic stimulative effect in the presence of extracts E_1, E_2 and of the fractions B and C was observed. No stimulative effect was detected in treatments with fractions A. The fraction B of each extract induced a significant ($P < 0.01$) dose-dependent intrinsic stimulation of the lymphoproliferative response (Fig. 4).

An active mitogen-extract interation was also observed in variations of mitogen-induced blastogenesis. The fraction B from both extracts produced a clear dose-dependent inhibition of the Con A-activation of mice splenocyte cultures; it may be a consequence of binding of ConA to mannose residues [20] in the bifidobacteria B fractions. The effect in the LPS-activation was particularly evident for the fraction B from E_1. Fraction C did not affect the ConA-induced blastogenesis but a slight stimulatory effect was observed when this fraction interacted with LPS (Fig. 5).

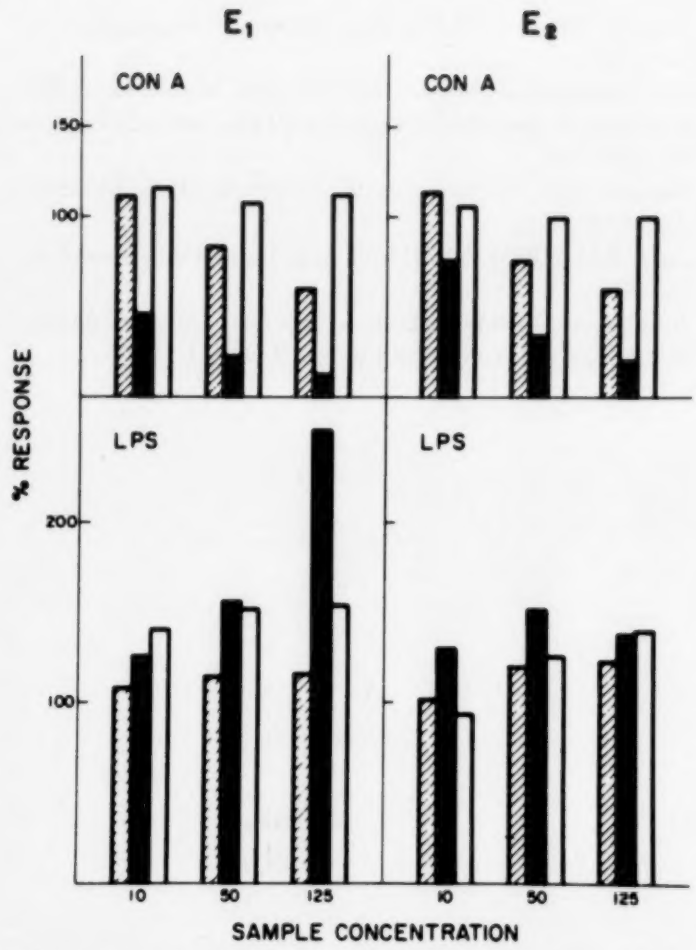

Fig. 5. Variations in the murine splenocyte response induced by 'in vitro' treatments with association of either Con A or LPS with the extracts E_1 and E_2 (▨) and their fraction B (■) and C (□). Results are given as % of the response of splenocytes treated with Con A or LPS. Net [^{3}H]-thymidine incorporation in control cultures was 194363 ± 11661 (Con A) and 27767 ± 1698 (LPS) cpm.

The effects of fractions A were not statistically significant (not shown).

It was reported that indigenous microbiota of the gastrointestinal tract of mice could contribute to the resistance of these animals against exogenous infections [21]. This resistance could be developed through the influence that the intestinal flora may exert on the organs involved in the immune response, as observed by Van der Waaij [22]. A similar effect may be attributed to the intestinal bifidobacteria.

Regarding the identification of the bifidobacterial components with immunodificative ability [2,9,10,23] our results suggest the carbohydrate-rich components may be responsible for modification of the immune response. This could take place as a consequence of their interaction with intestinal epithelial cells, as reported for the bifidobacterial lipoteichoic acid [2]. They could also release active mediators from gut-associated immunocompetent cells [22] as, for example, certain amphiphilic compounds [24]. Finally these mediators may modify immunological activities of the suppressor or helper T-cell subsets [20,25] or even at a cytotoxic response level [26]. Further experiments are in process for revealing the mechanisms of action of these compounds.

ACKNOWLEDGEMENTS

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REFERENCES

[1] Muting, D., Eschrich, W. and Mayer, J.B. (1968) Am. J. Proctol. 19, 336–342.
[2] Op den Camp, H.J.M., Oosterhof, A. and Veerkamp, J.H. (1985) Infect. Immun. 47, 332–334.
[3] Savage, D.C. (1970) Am. J. Clin. Nutr. 23, 1495–1501.
[4] Mata, L.J., Urrutia, J.J., García, B., Fernandez, R. and Behar, M. (1969) Am. J. Dis. Child. 117, 142–146.
[4] Harrison, A.P. Jr. and Mansen, P.A. (1954) Zentralb. Bakteriol. Parasitenk. Infectionskr Hyg. Abt. Orig. 161, 125–137.
[6] Sgorbati, B. and Scardoni, V. (1978) Antonie van Leeuwenhoek 45, 129–140.
[7] Sgorbati, B. (1979) Antonie van Leeuwenhoek 45, 557–564.
[8] Scardovi, V., Casalichio, F. and Vincenzi, N. (1979) Int. J. Syst. Bacteriol. 29, 312–327.
[9] Commins, C.S. (1984) in: Immune modulation agents and their mechanisms (Fernichel, R.L. and Chirigos, M.A. eds.), pp. 163–190.
[10] Breuillaud, J., Lemonie, A., Bertrand, M.A. (1976) Rev. Inst. Pasteur,Lyon 9, 213–232.
[11] Op den Camp, H.J.M., Peeters, P.A.M., Oosterhof, A. and Veerkamp, J.H. (1985) J. Gen. Microbiol. 131, 661–668.
[12] Lowry, O.H., Rosebrough, N.L., Far, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Trevelyan, W.E. and Harrison, J.S. (1972) Biochem. J. 50, 298–303.
[14] Laemmli, U.K. (1970) Nature (London) 227, 680–685.
[15] Laine, R.A., Esselman, W.J. and Sweeley, C.C. (1972) Methods Enzymology 28, 159–167.

[16] Ruperez, P. and Leal, J.A. (1981) Trans. Br. Mycol. Soc. 77, 621–625.
[17] Portolés, A., Ojeda, G., Rojo, J.M., Portolés, M.P. and Barasoain, I. (1983) J. Immunopharmacol. 5, 245–256.
[18] Melgar, M.M., Pérez Silva, G., Gil, I., Gomez, E. and Portolés, A. (1985) Rev. Esp. Fisiol. 41, 225–234.
[19] Rojo, J.M., Ojeda, G. and Portolés, M.P. (1984) Int. J. Immunopharmacol. 6, 61–65.
[20] Lis, H. and Sharon, N. (1986) Ann. Rev. Biochem. 55, 35–67.
[21] Roach, S. and Tannock, G.W. (1980) Can. J. Microbiol. 26, 408–411.
[22] Van der Waaij, D. (1986) Med. Microbiol. Immunol. 175, 335–340.
[23] De Simone, C., Ferrazzi, M., Di Seri, M., Mongio, F., Baldinelli, L. and Di Fabio, S. (1987) Int. Immunotherapy III, 151–158.
[24] Wicken, A.J. and Knox, K.W. (1980) Biochim. Biophys. Acta 604, 1–26.
[25] Berg, R.D. (1985) EOS J. Immunol. Immunopharmacol. 4, 161–165.
[26] Bartizal, K.F., Salkowski, C., Pleasants, J.R. and Balish, E. (1984) J. Leukocyte Biology 36, 739–743.

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Cloning of a maltogenic alpha-amylase from *Bacillus stearothermophilus*

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Key words: Maltogenic amylase; Cloning; Sequence; *Bacillus subtilis*

1. SUMMARY

We have cloned and expressed a novel maltogenic alpha-amylase from *B. stearothermophilus* on plasmid in *B. subtilis*. Originally the plasmid was very unstable in the absence of selection, but was stabilized due to a spontaneous, copy number reducing mutation. The promoter region and the extension of the gene have been analysed, and a provisional DNA sequence has been determined. The N-terminal of the mature amylase has been determined and shown to be in accordance with signal peptidase processing after a typical Gram-positive signal sequence of 33 amino acids.

2. INTRODUCTION

Starch is produced in large amounts from corn, cereals, potatoes, sorghum, and other plants. It is consumed by humans as well as animals, and is further used as a valuable raw material for the production of glucose, fructose, oligosaccharides, and alcohol.

Starch is a polymer of D-glucose units linked by 1,4-alpha-glucosidic bonds and occasionally 1,6-alpha-glucosidic bonds. Actual industrial processes use heat-stable alpha-amylase for degrading starch into a mixture of oligosaccharides which in its turn can be further degraded by other enzymes to obtain glucose.

Another starch degradation product of interest is the disaccharide maltose, principally because of its mild sweetness, stability, low hygroscopicity, and little tendency to crystallize [1]. It was therefore of interest to find an enzyme which, under industrial conditions and with high efficiency, could convert starch or oligosaccharides into maltose. Such a maltogenic amylase was identified in a *Bacillus stearothermophilus* strain [2]. However, the low enzyme productivity of this strain excluded commercial production of the amylase, and we therefore cloned *amyM*, the gene for the maltogenic amylase, and expressed it in *B. subtilis* as described in this publication. Furthermore, we analyzed the gene by DNA sequencing and pro-

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moter analysis and compared the amino acid sequence with other amylases.

3. MATERIALS AND METHODS

3.1. Bacterial strains and plasmids

The donor strain C 599 is a soil isolate from Krisuvik, Iceland. It is aerobic, rod shaped, spore forming, and grows between 50 °C and 70 °C. Accordingly, it was classified as belonging to the *B. stearothermophilus* group. *Escherichia coli* K-12 No. 802 is *met* r m^+ *gal lac* [3]. All *B. subtilis* strains are derivatives of *B. subtilis* 168. DN304 (*aroI906 sacA321 amyE*) is a Met^+ transformant of QB1133 [4]. DN314 is DN304/pUB110. Plasmids pACYC184, pUB110, and pBD64 have all been described [5–7].

3.2. Transformation

Competent *E. coli* and *B. subtilis* cells were prepared and transformed by established procedures [8,9].

3.3. Selection and screening

E. coli transformants were selected with 20 μg/ml chloramphenicol and *B. subtilis* transformants with 6 μg/ml chloramphenicol or 10 μg/ml kanamycin, in all cases on starch containing LB agar. Amylase positive transformants were identified upon exposure of the agar plate to iodine vapour.

3.4. Plasmid copy number

Plasmids were prepared by the alkaline method of Birnboim and Doly [10]. Relative plasmid copy number was estimated on agarose gels stained with ethidium bromide.

3.5. DNA sequencing

DNA was sequenced according to the Maxam-Gilbert method [11].

4. RESULTS

4.1. Cloning in E. coli

The *amyM* gene of *B. stearothermophilus* was firstly cloned in *E. coli* No. 802 using partially *Sau*3A degraded chromosomal DNA of 4–12 kb from *B. stearothermophilus* C599 ligated with *Bam*H1 cut and alkaline phosphatase treated pACYC184.

Among approx. 600 Cam^R Tet^S (chloramphenicol resistant and tetracycline sensitive) transformants, one was Amy^+ (amylase positive) and harboured plasmid pDN400 of 15 kb including the *amyM* gene.

4.2. Cloning in B. subtilis

pDN400 was partially digested by *Sau*3A, ligated with *Bam*H1 cut pBD64, and transfered into *B. subtilis* DN314, selecting for Cam^R (DN314 harbours pUB110 which serves as a rescue vector to increase the transformation efficiency of pBD64 [7]). An Amy^+ transformant was identified, and plasmid was prepared from this and transferred into *B. subtilis* DN304 which does not contain a rescue plasmid. An Amy^+ transformant, DN463, was identified and shown to harbour the recombinant plasmid pDN452 of 7.6 kb (Fig. 1); but not the rescue plasmid pUB110 of DN314.

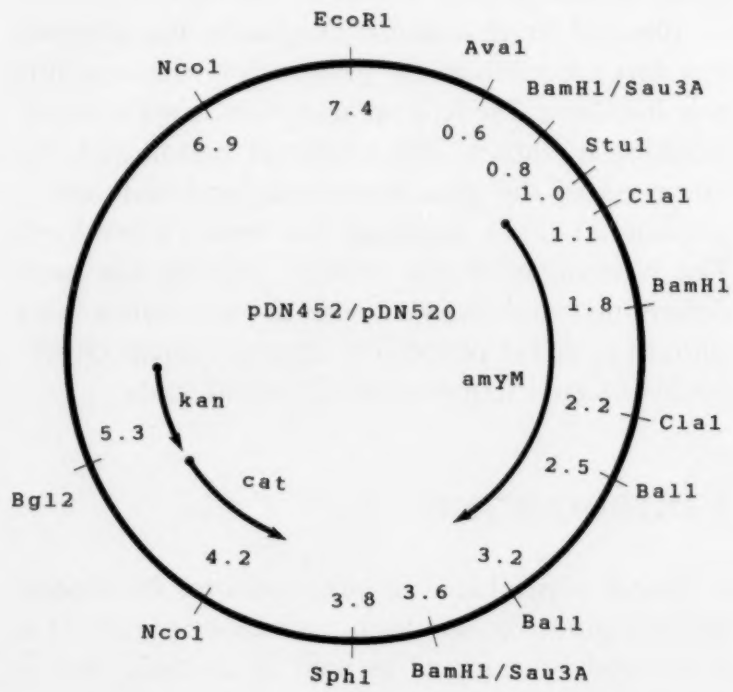


Fig. 1. Restriction map of plasmids pDN452 and pDN520. As described in the text (section 4.5), pDN520-20 was constructed from pDN520 by deletion of approx. 950 bp between the *Eco*R1 site and position 0.9 kb on the map.

4.3. Plasmid stability

B. subtilis DN 463/pDN452 looses its plasmid quickly in the absence of Cam and Kan (kanamycin). By repeated inoculation with rare Amy^+ colonies from cultures grown without antibiotics, a strain, DN520, with a significantly improved plasmid stability was isolated. Thus, more than 90% of DN520 grown overnight in a shake flask without kanamycin had retained plasmid pDN520 while less than 10% of DN463 still contained pDN452. The increased stability of DN520 was due to a mutation, *cop-520,* on the plasmid which was shown to reside between the *Nco*1 and *Eco*R1 sites at positions 6.9 and 7.4 kb, respectively (Fig. 1). *cop-520* causes an apparent ten fold reduction of the plasmid copy number which may be the cause of the increased stability. If the above fragment of other pUB110 derived plasmids is replaced by the corresponding fragment of pDN520 (including *cop-520*), the copy number of the recombinant is reduced approximately ten fold (results not shown). pDN452 has approximately the same copy number as pUB110, as estimated on agarose gels (see section 3.4).

4.4. Analyses of amyM

pDN520 harbours 2.8 kb cloned DNA including the *amyM* gene, and was found to encode a maltogenic amylase which is indistinguishable from the amylase of the *B. stearothermophilus* strain C599 with respect to pI (= 8.5), molecular mass (= 75 kDa), immunological properties, optimal activity (pH 5.5 and 60 °C), and enzymatic properties including the novel ability to hydrolyse maltotriose to maltose and glucose [2]. The molecular weight is equivalent to a DNA fragment of approx. 2.1 kb (not including signal peptide, ribosome binding site, and promoter). Thus, very little DNA unrelated to the *amyM* gene has been cloned on pDN520.

The amylase produced by *B. subtilis* strain DN520 is exported into the medium and represents the dominating protein band in a supernatant sample analysed by SDS-PAGE. No cell bound or unprocessed amylase has been identified.

4.5. DNA sequence analysis

To facilitate sequencing and analysis, pDN520 was reduced in size by *Bal*31 exonuclease treatment in the *Ava*1 site (Fig. 1) followed by addition of an *Eco*R1 linker, cutting by *Eco*R1, ligation, transformation into *B. subtilis,* and analyses of Amy^+ and Amy^- transformants. Removal of more than approx. 150 bp of the cloned material destroyed the Amy^+ phenotype. One plasmid which had lost approx. 140 bp and contained the *Eco*R1 linker was named pDN520-20 and used for further analysis.

The sequencing strategy is shown in Fig. 2, and the DNA sequence in Fig. 3. Note that only part of the gene has been sequenced in both directions.

Analysis of the DNA sequence shows an open reading frame of 717 amino acids including an N-terminal sequence with the properties of a typical gram-positive signal sequence. Preceding the N-terminal methionine, a typical ribosome binding site and a potential $-35-10$ promoter site were identified. The sequence data are in agreement with the following.

1. The calculated molecular weight of the amylase agrees with the observed value.
2. The calculated amino acid composition agrees with the one observed (results not shown).

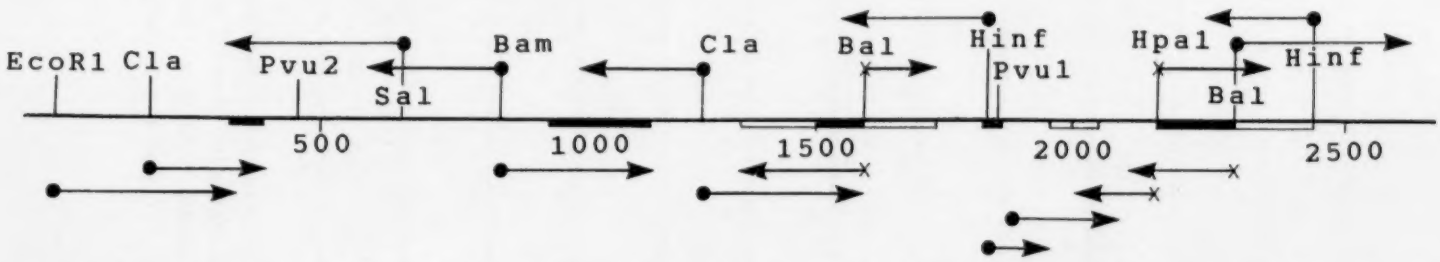


Fig. 2. Sequencing strategy of the *amyM* gene. Arrows with a dot represent 3′ labelled sequencing. Arrows with a cross represent 5′ labelled sequencing. An open bar shows that the region has been sequenced in both directions on the same strand. A closed bar shows that the region has been sequenced on both strands.

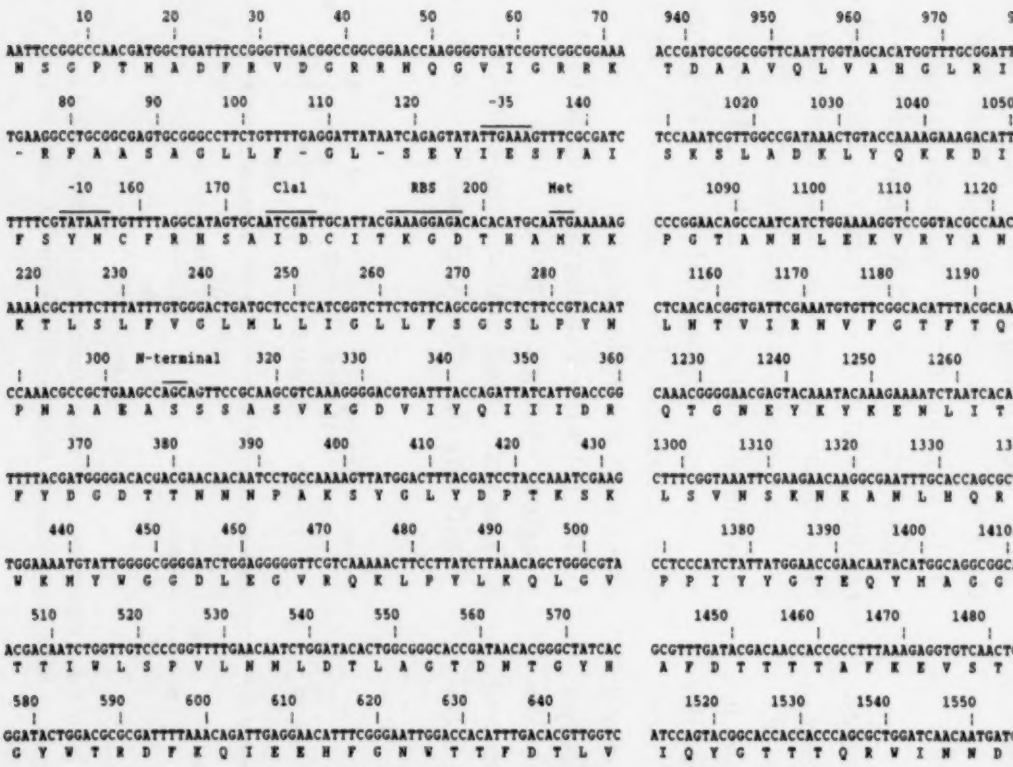
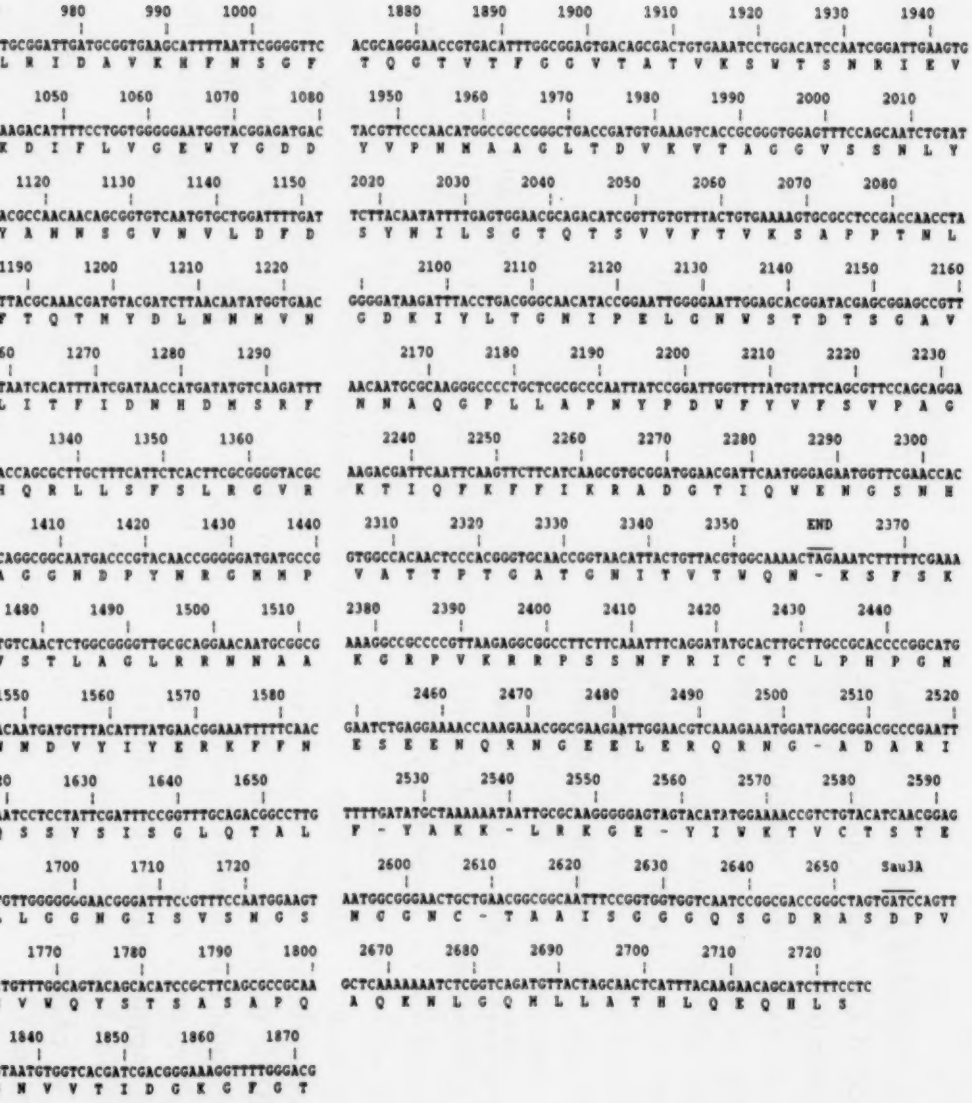

10 20 30 40 50 60 70
AATTCCGGCCCAACGATGGCTGATTTCCGGGTTGACGGCCGGCGGAACCAAGGGGTGATCGGTCGGCGGAAA
N S G P T M A D F R V D G R R N Q G V I G R R K
80 90 100 110 120 -35 140
TGAAGGCCTGCGGCGAGTGCGGGCCTTCTGTTTGAGGATTATAATCAGAGTATATTGAAAGTTCGCGATC
- R P A A S A G L L F - G L - S E Y I E S F A I
-10 160 170 ClaI RBS 200 Met
TTTTCGTATAATTGTTTTAGGCATAGTGCAATCGATTGCATTACGAAAGGAGACACATGCAATGAAAAAG
F S Y N C F R H S A I D C I T K G D T H A M K K
220 230 240 250 260 270 280
AAAACGCTTTCTTTATTTGTGGGACTGATGCTCCTCATCGGTCTTCTGTTCAGCGGTTCTCTTCCGTACAAT
K T L S L F V G L M L L I G L L F S G S L P Y M
300 N-terminal 320 330 340 350 360
CCAAACGCCGCTGAAGCCAGCAGTTCCGCAAGCGTCAAAGGGGACGTGATTTACCAGATTATCATTGACCGG
P N A A E A S S S A S V K G D V I Y Q I I I D R
370 380 390 400 410 420 430
TTTTACGATGGGGACACGACGAACAACAATCCTGCCAAAAGTTATGGACTTTACGATCCTACCAAATCGAAG
F Y D G D T T N N N P A K S Y G L Y D P T K S K
440 450 460 470 480 490 500
TGGAAAATGTATTGGGGCGGGGATCTGGAGGGGGTTCGTCAAAAACTTCCTTATCTTAAACAGCTGGGCGTA
W K M Y W G G D L E G V R Q K L P Y L K Q L G V
510 520 530 540 550 560 570
ACGACAATCTGGTTGTCCCCGGTTTTGAACAATCTGGATACACTGGCGGGCACCGATAACACGGGCTATCAC
T T I W L S P V L N N L D T L A G T D N T G Y N
580 590 600 610 620 630 640
GGATACTGGACGCGCGATTTTAAACAGATTGAGGAACATTTCGGGAATTGGACCACATTTGACACGTTGGTC
G Y W T R D F K Q I E E H F G N W T T F D T L V
660 670 680 690 700 710 720
AATGATGCTCACCAAAACGGAATCAAGGTGATTGTCGACTTTGTGCCCAATCATTCGACTCCTTTTAAGGCA
N D A H Q N G I K V I V D F V P N H S T P F K A
730 740 750 760 770 780 790
AACGATTCCACCTTTGCGGAAGGCGGCGCCCTCTACAACAATGGAACCTATATGGGCAATTATTTTGATGAC
N D S T F A E G G A L Y N N G T Y M G N Y F D D
800 810 820 830 840 850 860
GCAACAAAAGGGTACTTCCACCATAATGGGGACATCAGCAACTGGGACGACCGGTACGAGGCGCAATGGAAA
A T K G Y F H H N G D I S N W D D R Y E A Q W K
870 880 890 900 910 920 930
AACTTCACGGATCCAGCCGGTTTCTCGCTTGCCGATTTGTCGCAGGAAAATGGCACGATTGCTCAATACCTG
N F T D P A G F S L A D L S Q E N G T I A Q Y L
940 950 960 970
ACCGATGCGGCGGTTCAATTGGTAGCACATGGTTTGCGGATT
T D A A V Q L V A H G L R I
1020 1030 1040 1050
TCCAAATCGTTGGCCGATAAACTGTACCAAAAGAAAGACATT
S K S L A D K L Y Q K K D I
1090 1100 1110 1120
CCCGGAACAGCCAATCATCTGGAAAAGGTCCGGTACGCCAAC
P G T A N H L E K V R Y A N
1160 1170 1180 1190
CTCAACACGGTGATTCGAAATGTGTTCGGCACATTTACGCAA
L N T V I R N V F G T F T Q
1230 1240 1250 1260
CAAACGGGGAACGAGTACAAATACAAAGAAAATCTAATCACA
Q T G N E Y K Y K E N L I T
1300 1310 1320 1330
CTTTCGGTAAATTCGAAGAACAAGGCGAATTTGCACCAGCGC
L S V N S K N K A N L H Q R
1380 1390 1400 1410
CCTCCCATCTATTATGGAACCGAACAATACATGGCAGGCGGC
P P I Y Y G T E Q Y N A G G
1450 1460 1470 1480
GCGTTTGATACGACAACCACCGCCTTTAAAGAGGTGTCAACT
A F D T T T A F K E V S T
1520 1530 1540 1550
ATCCAGTACGGCACCACCACCCAGCGCTGGATCAACAATGAT
I Q Y G T T T Q R W I N N D
1590 1600 1610 1620
GATGTCGTGTTGGTGGCCATCAATCGAAACACGCAATCCTCC
D V V L V A I N R N T Q S S
1660 1670 1680 1690
CCAAATGGCAGCTATGCGGATTATCTGTCAGGGCTGTTGGGG
P N G S Y A D Y L S G L L G
1740 1750 1760 1770
GTCGCTTCGTTCACGCTTGCGCCTGGAGCCGTGTCTGTTTGG
V A S F T L A P G A V S V V
1810 1820 1830 1840
ATCGGATCGGTTGCTCCAAATATGGGGATTCCGGGTAATGTG
I G S V A P N N G I P G N V

Fig. 3. DNA sequence of the *amyM* gene and flanking regions. Bases 1–2658 originate fro
pUB110 flanking the *Bam*H1 site [12]. At positions 128 and 151, typical −35 and −1
typical ribosome binding and translation initiation sequences were found. A typical signal
at position 307, and at position 2359 the 684 amino acid mature protein is most likely te



ate from *B. stearothermophilus,* and bases 2659–2726 are identical to the sequence of
d −10 RNA polymerase binding sequences have been identified. At 189 and 208,
signal sequence of 33 amino acids is predicted to be processed between Ala and Ser
kely terminated.

amy L:	90	L	H	S	R	D	I	N	V	Y	G	D	V	V	I	N	H	K
amy S:	91	A	H	A	A	G	M	Q	V	Y	A	D	V	V	F	D	H	K
amy M:	117	A	H	Q	N	G	I	K	V	I	V	D	F	V	P	N	H	S
amy E:	87	A	E	E	Y	G	I	K	V	I	V	D	A	V	I	N	H	T

amy L:	225	L	D	G	F	R	L	D	A	V	K	H	I
amy S:	228	I	D	G	F	R	L	D	A	V	K	H	I
amy M:	219	A	H	G	L	R	I	D	A	V	K	H	F
amy E:	170	A	D	G	F	R	F	D	A	A	K	H	I

amy L:	322	V	T	F	V	D	N	H	D	T
amy S:	324	V	T	F	V	D	N	H	D	T
amy M:	319	I	T	F	I	D	N	H	D	M
amy E:	262	V	T	W	V	E	S	H	D	T

Fig. 4. Amino acid homology in three conserved regions [14] between *amyM* (B. stearothermophilus, this work), *amyL* (B. licheniformis, [15]), *amyS* (B. stearothermophilus, (15)), and *amyE* (B. subtilis, (16)). Numbers refer to the position relative to the N-terminal of the mature amylases.

3. The *Eco*R1-*Cla*1 fragment of 176 bp has strong promoter activity (see below).
4. Deletion of the last 60 amino acids (predicted from the sequence) significantly reduces amylase activity (unpublished results).

According to von Heijne [13], the signal cleavage is most likely to occur after amino acid 33, leaving a Ser-Ser-Ser-Ala-Ser-Val N-terminal on the mature amylase (Fig. 3). N-terminal determination of the mature amylase purified from *B. subtilis* DN520/pDN520 gave the sequence: Ser-x-x-Ala-x-Val-x-Gly-Asp-Val-Ile-Tyr which is in accordance with the prediction.

The deduced amino acid sequence shows significant homology with other amylases in three regions typical to these enzymes [14] (Fig. 4).

4.6. Promoter activities

The 176 bp *Eco*R1-*Cla*1 fragment, which contains a typical −10 and −35 RNA polymerase binding region, was cloned on plasmid pDN1380 (Fig. 5). Upon insertion of *kanD,* which is a promoterless kanamycin resistance gene (see Fig.

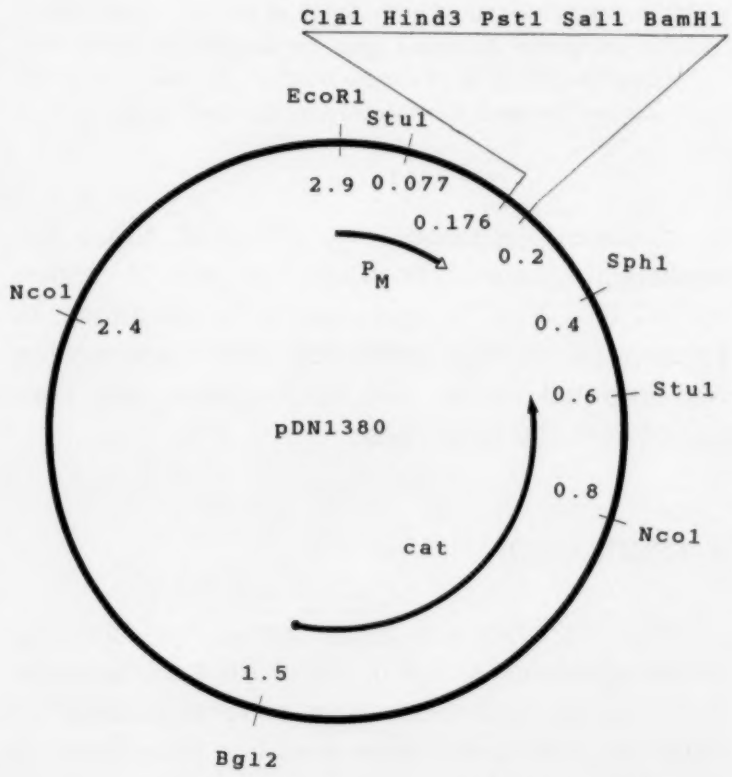


Fig. 5. Restriction map of pDN1380. pDN1380 was constructed by inserting the 176 bp *Eco*R1-*Cla*1 fragment from *amyM* into cloning vector pDN1050 [17]. P_M signifies the promoter from *amyM*.

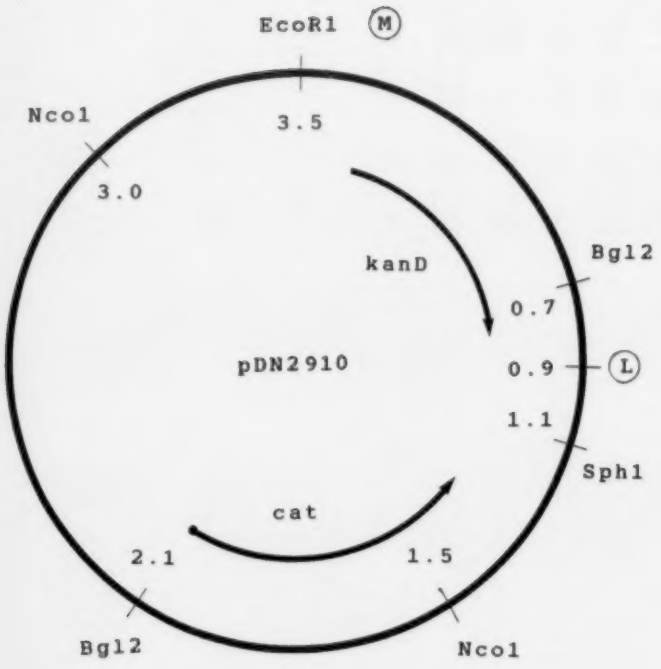


Fig. 6. Restriction map of plasmid pDN2910. M and L symbolize the following multilinkers, read clockwise: (M) *Cla*1, *Hin*d3, *Pst*1, *Sal*1, *Bam*H1, *Sma*1, *Kpn*1, *Sac*2, *Xma*3, *Pvu*1, *Apa*1, *Sac*1, *Kpn*1. (L) *Kpn*1, *Sma*1, *Bam*H1, *Xba*1, *Sal*1, *Hin*d3. *kan*D is a promoterless derivative of the *kan*A gene of *Staphylococcus aureus* plasmid pUB110. Referring to the sequence of pUB110 [12], *kan*D starts with basepair number 3015 in an *Ssp*1 site which has been fused with *Sma*1 of *E. coli* plasmid pUC19, and stops at number 2164 in an *Sna*B1 site which has also been fused with *Sma*1 of pUC19. Thus, *kan*D includes the entire structural gene for kanamycin nucleotidyl transferase including its ribosome binding site, but neither the promoter nor the gene for phleomycin resistance [12].

6), a strong resistance was obtained. Since the equivalent plasmid without the *amyM* region (pDN2910, Fig. 6) did not give resistance to kanamycin, it was concluded that transcription was initiated in the 176 bp fragment and continued into the *kanD* gene.

5. DISCUSSION

New enzymes are often found by screening microorganisms in soil or water samples. Usually, however, enzyme yields from natural isolates are very low, and an extensive mutation programme is therefore needed to obtain a strain which is useful from a commercial point of view. Modern gene techniques offer the possibility of bypassing such a time consuming and often unrewarding project.

The cloning of a maltogenic amylase and stabilization of its expression from a plasmid in *B. subtilis* described in this paper have allowed high-yield expression of the amylase and permitted demonstration of its application potential [1,2]. An alternative method for plasmid stabilization by suppression of a D-alanine requirement has previously been described [17] and exemplified using the maltogenic amylase gene.

High amylase yields were obtained using the native promoter of the *amyM* gene. With a new, useful promoter probing gene, *kanD,* it was demonstrated that a small region preceding the structural *amyM* gene included an efficient promoter.

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REFERENCES

[1] Slominska, L. and Starogardzka, G. (1986) Starch 38, 205–210.
[2] Outtrup, H. and Norman, B.E. (1984) Starch 36, 405–411.
[3] Wood, B. (1966) J. Mol. Biol. 16, 118–133.
[4] Steinmetz, M., Kunst, F. and Dedonder, R. (1976) Mol. Gen. Genet. 148, 281–285.
[5] Chang, A.C.Y. and Cohen, S.N. (1978) J. Bacteriol. 134, 1141–1156.
[6] Gryczan, T.J., Contente, S. and Dubnau, D. (1978) J. Bacteriol. 134, 318–329.
[7] Gryczan, T.J., Shivakumar, A.G. and Dubnau, D. (1980) J. Bacteriol. 141, 246–253.
[8] Mandel, M. and Higa, A. (1970) J. Mol. Biol. 53, 159–162.
[9] Yasbin, R.E., Williams, G.A. and Young, F.E. (1975) J. Bacteriol. 121, 296–304.
[10] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1512–1522.
[11] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–566.
[12] McKenzie, T., Hoshino, T., Tanaka, T. and Sueoka, N. (1986) Plasmid 15, 93–103.

[13] Von Heijne, G. (1983) Eur. J. Biochem. 133, 17–21.
[14] Friedberg, F. (1983) FEBS Lett. 152, 139–140.
[15] Gray, G.L., Mainzer, S.E., Rey, M.W., Lamsa, M.L., Kindle, K.L., Carmona, C. and Requadt, C. (1986) J. Bacteriol. 166, 635–643.
[16] Yang, M., Galizzi, A. and Henner, D. (1983) Nucleic Acids Res. 11, 237–249.
[17] Diderichsen, B. (1986) in Bacillus Molecular Genetics and Biotechnology Applications (Ganesan, A.T. and Hoch, J.A., eds.), pp. 35–46, Academic Press.

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Behaviour of Nif⁻ mutants of *Rhodobacter capsulatus* in photosynthetic, ammonia-limited chemostat cultures

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1. SUMMARY

Nif⁻ mutants of *Rhodobacter capsulatus* carrying mutations either in the *nifR4* regulatory gene or in the *nifH* structural gene both outgrew the wild-type strain B10 in mixed chemostat cultures under conditions favouring nitrogenase-mediated H_2 production by the wild-type (ammonia as limiting nutrient, inert argon atmosphere, light as energy source), whereas under aerobic conditions in the dark, or in batch culture, the growth of Nif⁻ mutants was not favoured. Nitrogenase-mediated H_2 production therefore appears to be detrimental to the growth of *R. capsulatus* in nitrogen-limited continuous culture, as may also be the case for other nitrogen fixers.

2. INTRODUCTION

Nitrogen fixation is known to be a highly energy requiring process, although the exact energy requirement of nitrogen fixation is difficult to determine, and may vary from one species to another (see, e.g. [1,2] for reviews). A number of factors contribute to the energy cost of nitrogen fixation, including the ATP-dependent reduction of N_2 to NH_3 by nitrogenase, the retention of NH_3, the assimilation of NH_3 via glutamine synthetase, and the production of H_2 by nitrogenase, which occurs concomitantly with N_2 fixation. When nitrogenase synthesis is derepressed in the absence of N_2, H_2 production by nitrogenase also occurs. This process is particularly active in the photosynthetic purple, non-sulphur bacteria, such as *Rhodobacter capsulatus,* where it is known as H_2 photoproduction [3,4].

In the purple, non-sulphur bacteria, it has been postulated that nitrogenase-mediated H_2 production may play a physiological role as an 'electron sink', allowing the dissipation of the excess reducing equivalents produced under conditions of nitrogen-deprivation [5]. However, Wall et al. [6] found that, when a streptomycin-resistant strain of *R. capsulatus* was subcultured repeatedly in a nitrogenase-derepressing (malate-glutamate) medium, spontaneous Nif⁻ mutants accumulated, eventually reaching a proportion of greater than 99% of the total cell population. Spontaneous Nif⁻ mutants also arose in nitrogen-limited chem-

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ostat cultures of the wild-type strain B10, but in this case, the proportion of Nif^- mutants in the culture stabilised at values between 20% and 50% of the total cell population [7].

The majority of spontaneous Nif^- mutants of *R. capsulatus,* including those isolated in chemostat culture, contained mutations which mapped in a single *nif* regulatory gene, *nifR4* [6,8–10]. These mutants were characterized by a lack of detectable synthesis of nitrogenase protein [6,8] and *nifH::lacZ* gene fusions were not expressed in these mutants [9]. Since these mutants were regulatory mutants, their appearance in nitrogen-limited cultures might have been due either to the lack of nitrogenase synthesis and activity, or to an additional effect of the regulatory mutation which favoured growth under nitrogen-limited conditions. In order to distinguish between these two possibilities, we have examined the behaviour of both a regulatory *nifR4* mutant and a structural *nifH* mutant in mixed chemostat cultures with the wild-type. We have also studied the behaviour in chemostat culture of a glutamine auxotroph, G29, which is depressed for nitrogenase synthesis in the presence of NH_4^+, and of an Ntr^- mutant, RC34, which is pleiotropically deficient in growth on N sources other than NH_4^+. The results of these studies indicate that the lack of nitrogenase synthesis and activity is sufficient to explain the appearance of Nif^- mutants in nitrogen-limited continuous culture, and that nitrogenase-mediated H_2 production is therefore detrimental to growth of the wild-type under these conditions.

3. MATERIALS AND METHODS

3.1. Bacteria and growth conditions

The wild-type strain used was B10 [8,11], and the mutants used were derived from this strain. The spontaneous Nif^- mutant, AP29, was isolated from a glutamate-limited chemostat culture [7] and carries a revertible mutation in the *nifR4* regulatory gene [8]. The Nif^- mutant, RC33, was isolated after chemical mutagenesis [12] and carries a point mutation in the *nifH* gene which is polar onto *nifD* and *nifK* [8]. The Ntr^- (nitrogen regulation) mutant, RC34, is unable to utilise individual amino acids or N_2 as sole N source for growth, but grows at similar rates to the wild-type on NH_4^+ [8,13]. The glutamine (Gln^-) auxotroph, G29, which is fully derepressed for nitrogenase synthesis and activity in the presence of NH_4^+ [14] was kindly provided by Dr J.D. Wall, University of Columbia, Columbia, MO, USA.

Pre-cultures of different strains were grown photosynthetically in RCV medium [3,15] containing 40 mM DL-malate as carbon source and 7.5 mM $(NH_4)_2SO_4$ as nitrogen source. For growth of G29, this medium was supplemented with 12 mM glutamine. Photosynthetic continuous cultures were grown under argon in a New Brunswick Bioflo C30 fermenter, as described previously [9], using an RCV medium containing 25 mM DL-lactate as carbon source and 5 mM NH_4Cl as nitrogen source. For dark, aerobic growth, the culture vessel was covered in aluminium foil, compressed air was blown through the culture at a rate of 1.5 l/h and the concentration of DL-lactate was increased to 50 mM to ensure nitrogen-limitation. The medium was also supplemented with polypropylene glycol 2000 (0.1%) to prevent foaming. For serial batch cultures, 100 ml of medium containing 25 mM DL-lactate and 5 mM NH_4Cl in a stoppered Erlenmeyer flask was gassed with argon and inoculated with 1 ml of a culture grown for 24 h in the light under the same conditions.

The phenotypic composition of cultures was determined as follows. Appropriate dilutions of a culture sample were plated on RCV agar medium to determine the total number of cells/ml. The number of Nif^+ cells/ml was then determined either by plating serial dilutions of the culture onto RCV agar medium without added nitrogen source (when the concentration of Nif^+ cells was less than 1%) or by replica-plating 100 to 200 colonies grown on RCV agar medium onto RCV agar medium without added nitrogen source. Agar plates were incubated in the light in Gas-Pak anaerobic jars (BBL, Cockeysville, MD, USA) under an atmosphere of $H_2 + N_2 + CO_2$, as described previously [12].

3.2. Analytical methods

Lactate was assayed enzymatically as described by Gutman and Wahlefeld [16] and the concentra-

tion of ammonia was determined colorimetrically [17]. H_2 production was measured by injecting samples from the gas phase into an Intersmat IGC120 gas chromatograph equipped with a thermal conductivity detector and Porapak Q column. Nitrogenase activity was estimated by measuring the rate of acetylene reduction in washed cell suspensions, as described previously [12]. Bacterial dry weights were calculated from the optical density of cultures at 660 nm, assuming that an $O.D._{660}$ of 1.0 is equivalent to 0.5 mg dry weight/ml [18].

4. RESULTS

The spontaneous *nifR4* mutant, AP29, was first grown photosynthetically in continuous culture, with ammonium as limiting nutrient, at a dilution rate (D) of 0.057 h^{-1}. In this experiment, the concentration of Nif^+ revertants increased from an initial value of 1×10^1/ml to a plateau value of 1×10^5/ml after 25 days of culture; this latter value corresponded to about 0.01% of the total cell population. If the dilution rate was then increased stepwise, over a period of 10 days, to 0.186 h^{-1}, the $O.D._{660}$ decreased from 2.5 to 1.5, but the concentration of Nif^+ cells remained constant.

The behaviour of AP29 was next studied in mixed culture with B10 (Fig. 1A). In this case, the proportion of Nif^- cells increased rapidly, from an initial value of 1%, to a value of around 95% after 5 days of culture. The nitrogenase activity in washed cells taken from the culture decreased to very low levels, although the decrease in activity appeared to lag behind the decrease in the proportion of Nif^+ cells. When the dilution rate was increased from 0.057 h^{-1} to 0.205 h^{-1} after 13 days, the $O.D._{660}$ of the culture decreased, and the proportion of Nif^+ cells increased abruptly to 25%, with a concomitant increase in nitrogenase activity and H_2 production (Fig. 1A).

In an experiment performed at a higher dilution rate ($D = 0.1$ h^{-1}, data not shown), the proportion of Nif^- mutants increased more slowly than at the lower dilution rate, but reached a level of 95% after 8 days of culture. In this experiment, however, the decrease in nitrogenase activity closely paralleled the decrease in the proportion of Nif^+ cells.

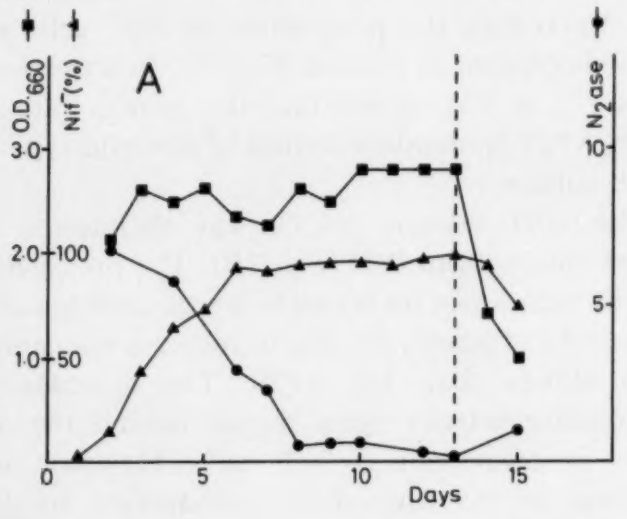
Finally, when strains AP29 and B10 were grown together for 10 days either aerobically in the dark in continuous culture, conditions under which nitrogenase synthesis does not occur, or photosynthetically in serial batch culture (see MATERIALS AND METHODS), the proportion of Nif^- cells remained constant at around 45 ± 5%. In a previous study [7], it was shown that the growth rate of strain AP29 is identical to that of the wild-type in batch culture.

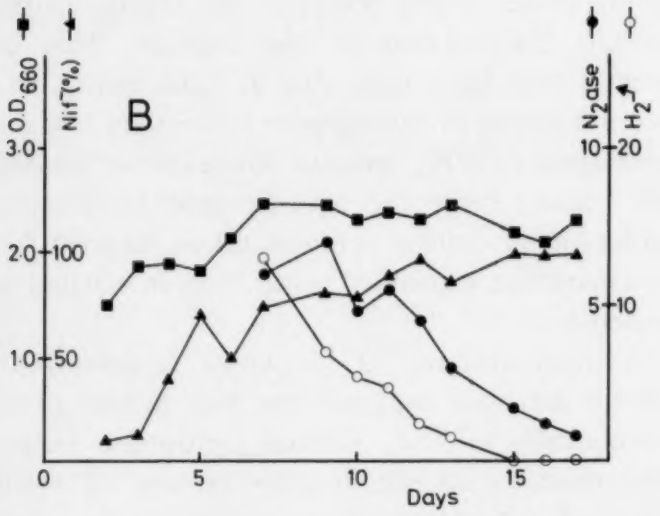
The *nifH* mutant, RC33, was also tested in mixed culture with B10 (Fig. 1B). The proportion of Nif^- cells again increased to a high level (greater than 99%), although the rate of increase was somewhat slower than for AP29. The decrease in nitrogenase activity again lagged behind the decrease in proportion of Nif^+ cells. However, the decrease in the rate of H_2 production by the culture more closely followed the change in phenotypic composition of the culture. This difference may have been due at least partly to in vivo inhibition of nitrogenase activity by low concentrations of NH_4^+ present in the culture medium (see below) since the specific rate of acetylene reduction by culture samples taken directly from the chemostat was much lower than in washed cell suspensions.

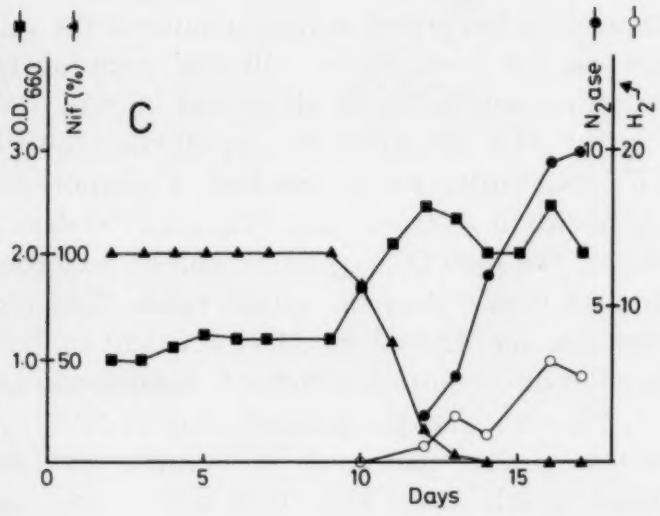
A third mutant, RC34, which is defective in general nitrogen metabolism, was grown photosynthetically in NH_4^+-limited continuous culture. This strain is unable to grow on any of a wide range of nitrogen sources, including N_2 (Ntr^- phenotype), but grows at similar rates to the wild-type on NH_4^+ in batch cultures, even at low concentrations [8,13]. In chemostat culture, however (Fig. 1C), this strain was rapidly outgrown by Nif^+ revertants, which reached a proportional concentration of more than 99% after 10 days of culture. The final $O.D._{660}$ of the culture was about two-fold higher than the initial value. This indicates that the mutant RC34 is deficient in NH_4^+ assimilation in nitrogen-limited continuous culture. Nevertheless, the residual level of NH_4^+ was similar to that measured in cultures of other strains, which varied from 0.08 mM to less than

0.02 mM, and showed no correlation with the ratio of Nif^+ and Nif^- cells.

In a final experiment, the glutamine (Gln^-) auxotroph, G29, which is derepressed for nitrogenase synthesis and activity in the presence of NH_4^+ (14), was grown photosynthetically in continuous culture ($D = 0.057\ h^{-1}$) in a medium containing 5 mM NH_4Cl, and 2.5 mM glutamine as N source. The Gln^- strain was rapidly outgrown by Gln^+ revertants, which reached a level of more than 99% of the total cell population after 10 days of culture. During this time, the nitrogenase activity in washed cell suspensions decreased to about 10% of its initial value.







5. DISCUSSION

The results presented above show that, in mixed chemostat cultures grown photosynthetically under nitrogen-limited conditions, strains which are deficient in nitrogenase synthesis have a selective advantage over strains which synthesise nitrogenase and produce H_2 under these conditions. An exception was the Ntr^- strain, RC34, which is apparently deficient in the assimilation of NH_4^+ at the low concentrations present in the chemostat. It therefore seems unlikely that spontaneous Nif^- mutants, such as AP29, are deficient in NH_4^+ assimilation, as suggested previously [7] and their appearance in chemostat cultures is probably related to the absence of nitrogenase synthesis in these strains. The difference in the rate of increase in the proportion of Nif^- cells between AP29 and the *nifH* mutant, RC33, may be due to the more drastic effect on *nif* gene expression in the regu-

Fig. 1. Phenotypic changes in photosynthetic, ammonia-limited chemostat cultures. The chemostat, containing 450 ml of growth medium containing 25 mM DL-lactate and 5 mM NH_4Cl, was inoculated with 5 ml each of precultures of the following strains: (A) AP29 (Nif^-) and B10 (wild-type); (B) RC33 (Nif^-) and B10 (wild-type); (C) RC34 (Nif^-/Ntr^-). The precultures were in the stationary phase of growth, at an $O.D._{660}$ of 3.0–4.0, and contained approx. 1×10^9 cells/ml. Cultures were grown overnight (12–16 h) in batch mode, and then grown in continuous culture mode for the number of days shown, at a dilution rate of 0.057 h^{-1}. In (A), the dilution rate was increased to 0.205 h^{-1} after 13 days (dotted line). Note that, in (A) and (B), the initial ratio of Nif^- to Nif^+ cells were very low, even though the cultures were inoculated with similar numbers of mutant and wild-type cells. 'N_2ase' indicates the specific nitrogenase activity of washed cell suspensions (nmol C_2H_2 reduced/min. mg cell dry wt.$^{-1}$). 'H_2↗' indicates the rate of H_2 production by the chemostat culture (nmol H_2 produced/min·mg cell dry wt^{-1}).

latory mutant than in RC33, in which only the expression of the *nifHDK* genes is expected to be affected. This would also explain why *nif* regulatory mutants are preferentially selected for in nitrogen-limited continuous culture.

Nitrogenase-mediated N_2 production, therefore, appears to be detrimental to the growth of *R. capsulatus* in nitrogen-limited continuous culture. Why this should be so, however, is unclear. Although the energy cost of H_2 production is high (see above), this does not explain the selection of Nif^- mutants unless nitrogen-limited cultures are also energy-limited [see 19]. An alternative explanation is that nitrogenase activity is inhibitory to NH_4^+ assimilation, an effect which might be mediated via the intracellular ATP concentration or redox state. This explanation does not apply to the Gln^+ mutant, G29, which is unable to assimilate NH_4^+. However, in this case, the Gln^+ revertants which accumulated were not N-limited, since NH_4^+ was present in excess in the growth medium, so they may have been energy-limited.

One approach to solving this problem would be to determine accurately the number of cellular units in cultures with different phenotypic compositions. If nitrogen-limited cultures of *R. capsulatus* are also energy-limited, then the cell density of cultures containing mainly Nif^- cells would be expected to be higher than in cultures containing mainly Nif^+ cells. The data obtained in the present study were insufficient to permit a statistical analysis, and it would also be desirable to determine cell numbers by cell counting, rather than the number of colony forming units, since *R. capsulatus* may grow in chains under some conditions [15]. As a general point, it would also be of interest to determine whether Nif^- mutants have a selective advantage in nitrogen-limited cultures of non-photosynthetic nitrogen fixers, or whether this phenomenon is specific to photosynthetic bacteria.

Two additional aspects of the data presented above require further discussion. Firstly, although the chemostat cultures shown in Figs. 1A and 1B were incubated with equal numbers of Nif^- and Nif^+ cells, the level of Nif^- cells after the first day of continuous culture was only about 1%. This suggests that the Nif^- mutants were slow to begin growth in the chemostat, although such a lag was not observed under dark, aerobic conditions, or in batch culture. Another possibility is that aggregates formed between Nif^- and Nif^+ cells, since such aggregates would give more rise to Nif^+ colony forming units. It seems unlikely that the Nif^- cells observed were derived from the wild-type cells, since the level of Nif^- cells was approx. 10% after 2 days of culture, whereas in continuous cultures of the wild-type the appearance of Nif^- mutants was detected only after 5 days culture [7].

A second point concerns the decrease in the nitrogenase activity of washed cell suspensions, which lagged behind the decrease in the proportion of Nif^+ cells. This lag was observed at $D = 0.057\ h^{-1}$, but not a higher dilution rates. We have no explanation for this lag, other than that a proportion of the Nif^+ cells become non-viable before being washed out of the chemostat. This possibility could be tested by determining the proportion of non-viable cells in the culture. A lack of correlation between the rate of H_2 production by chemostat cultures and the proportion of Nif^+ cells was observed in a previous study [7].

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REFERENCES

[1] Kleiner, D. (1985) FEMS Microbiol. Rev. 32, 87–100.
[2] Stam, H., Stouthamer, A.H. and van Verseveld, H.W. (1987) FEMS Microbiol. Rev. 46, 73–92.
[3] Hillmer, P. and Gest, H. (1977) J. Bacteriol. 129, 724–731.
[4] Vignais, P.M., Colbeau, A., Willison, J.C. and Jouanneau, Y. (1985) Adv. Microb. Physiol. 26, 155–234.
[5] Gest, H. (1972) Adv. Microb. Physiol. 7, 243–282.
[6] Wall, J.D., Love, J. and Quinn, S.P. (1984) J. Bacteriol. 159, 652–657.
[7] Allibert, P., Odom, M.J., Wall, J.D. and Vignais, P.M. (1984) FEMS Microbiol. Lett. 23, 221–226.
[8] Willison, J.C., Ahombo, G., Chabert, J., Magnin, J.P. and Vignais, P.M. (1985) J. Gen. Microbiol. 131, 3001–3015.
[9] Kranz, R.G. and Haselkorn, R. (1985) Gene 40, 203–215.

[10] Ahombo, G., Willison, J.C. and Vignais, P.M. (1986) Mol. Gen. Genet. 205, 442–445.
[11] Marrs, B. (1974) Proc. Natl. Acad. Sci. USA 71, 971–973.
[12] Willison, J.C. and Vignais, P.M. (1982) J. Gen. Microbiol. 128, 3001–3010.
[13] Allibert, P., Willison, J.C. and Vignais, P.M. (1987) J. Bacteriol. 169, 260–271.
[14] Wall, J.D. and Gest, H. (1979) J. Bacteriol. 137, 1459–1463.
[15] Weaver, P.F., Wall, J.D. and Gest, H. (1975) Arch. Microbiol. 105, 207–216.
[16] Gutmann, I. and Wahlefeld, A.W. (1974) Methods in Enzymatic Analysis (Bergmeyer, H.U., ed.) Vol. 3, pp. 1463–1468, Academic Press, London and New York.
[17] Weatherburn, M.W. (1967) Anal. Chem. 39, 971–974.
[18] Meyer, J., Kelley, B.C. and Vignais, P.M. (1978) J. Bacteriol. 136, 201–208.
[19] Dykhuizen, D.E. and Hartl, D.L. (1983) Microbiol. Rev. 47, 150–168.

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Improvements in the transformation of *Bacillus subtilis* protoplasts with plasmid DNA

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1. SUMMARY

The transformation system currently used for *Bacillus subtilis* protoplasts has been improved. Special emphasis was made on three parameters of practical importance:

(a) conditions for direct selection of transformants, (b) optimization of the transformation system for Rec$^-$ mutants, and (c) conservation of protoplast suspensions for further use.

Selective regeneration was efficiently achieved for kanamycin or neomycin. Chloramphenicol, tetracycline and erythromycin were only expressed when low concentrations of the antibiotics were used to select transformants during regeneration.

2. INTRODUCTION

Protoplast transformation induced by polyethylene glycol (PIP transformation) has been shown to be a very efficient technique to introduce plasmids in bacilli [1]. Developed as an alternative to plasmid transformation of competent cells in *Bacillus subtilis,* its efficiency is up to an order of magnitude higher than that of competent cells; up to 10% of the regenerants are transformed with 1 μg of DNA.

Protoplasts can be transformed by covalently closed monomeric plasmids [2] as well as by nicked monomeric forms and even single stranded DNA [3]. However, this technique is not routinely used since selection of transformants is laborious and involves manipulation of fragile protoplasts. This is specially true for Rec$^-$ protoplasts which revert poorly to the bacillary form; this fact has obstructed the use of these strains in transformation and fusion experiments.

In the present paper we describe improvements that render this technique as easy and efficient as the *Escherichia coli* transformation system. Modifications have been introduced that allow to efficiently select transformants during regeneration for most of the resistance markers commonly used. Also, improvements that make direct selection amenable to the transformation of Rec$^+$ and Rec$^-$ cells are reported, as well as an efficient method for the conservation of frozen protoplast preparations for further use.

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3. MATERIALS AND METHODS

3.1. Bacterial strains and plasmids

See Table 1.

3.2. Protoplast formation and regeneration

Protoplasts were obtained as described in [4] in SMM hypertonic buffer containing 200 μg/ml of lysozyme. Wall regenerations were performed by plating on hypertonic solid media; DM3 or mR are described in [1] and [5], respectively.

3.3. Transformation of protoplasts

A transformation protocol derived from [1] was utilized. Samples (0.5 ml) of the protoplast suspensions in SMM (2×10^8 protoplasts/ml) were mixed with 100 μl of the DNA solution and 0.5 ml of a 40% stock solution of PEG 6000 (Merck) in SMM. After 2 min of incubation at room temperature, dilutions were spread onto regeneration medium plates with (direct selection) or without (two step selection) the appropriate antibiotics. Replica plating was performed on nutrient antibiotic agar plates.

Plasmids were isolated according to the alkaline extraction method of Birboim and Doly [6] and purified by CsCl density gradients. pPL608 was digested to completion with *Pst*I (Pharmacia) and re-ligated with T4 DNA ligase according to Maniatis et al. [7].

Table 1

Bacterial strains and plasmids

B. subtilis	Relevant genotype or phenotype	Origin
Strains		
GSY1104	*purB34 ura-1 trpF7*	[4]
S13	derived from GSY1104 (but *rfm-486*)	[9]
GSY1035	*metC3 leu* Ery^R	[8]
GSY1277-3	*recE4* derivative of GSY1035	[8]
IA510	*recE4 r⁻ m⁻ stp*	[10]
Plasmids		
pC194 (2.9 kb)	Cm^R	[11]
pE194 (3.7 kb)	Em^R	[11]
pBC16 (4.2 kb)	Tc^R	[12]
pUB110 (4.5 kb)	Km^R or Neo^R, $Bleo^R$	[13]
pTV1 (12.4 kb)	Cm^R, (Em^R) Tn*917*	[14]
pPL608 (5.1 kb)	Cm^R, Neo^R expression vector replicon pUB110	[15]
pMK3 [1] (7.2 kb)	shuttle vector *E. coli, B. subtilis* (pUC8-pUB110) $Amp^R \cdot Km^R$	[16]

[1] DNA from plasmid pMK3 was obtained from *E. coli* JM83 [7].

Km = kanamycin; Neo-neomycin; Cm = chloramphenicol; Tc = tetracycline; Em = erythromycin.

4. RESULTS AND DISCUSSION

It has been previously shown that selection of resistance markers after transformation of protoplasts with plasmid DNA, required an incubation period in hypertonic rich buffer (SMMP) [1] necessary for cell-wall initiation and expression [16].

In an attempt to simplify the transformation protocol we assayed conditions which allowed the recovery of transformants without further manipulations for a variety of plasmids and strains. All the experiments were performed with protoplasts generated in SMM buffer because they were less fragile. As can be seen in Table 2A high values of transformants were obtained for the seven plasmids tested after direct or two step selection. However, the antibiotic concentration had to be considerably lowered to obtain chloramphenicol, tetracycline or erythromycin resistant clones. With this last antibiotic specially, great variations in the minimal concentration required were observed depending on the source. In contrast, kanamycin- (Km^R) or neomycin- (Neo^R) resistant transformants could be selected over a wide range of antibiotic concentration (20 to 50 μg/ml). For pPL608 difficulties in the establishment or replication of the plasmid seemed unlikely to explain this finding, since the kanamycin determinant carried on pPL608 was easily expressed while the chloramphenicol one was not. In contrast, low numbers of transformants were reproducibly obtained with pTV1, indicating difficulties in plasmid establishment as has been also reported by others [17].

Therefore, direct selection of transformants was as efficient as the two step procedure and often gave better results since the 'crowding effect' exerted by the untransformed protoplasts was

Table 2

Transformation efficiencies

Receptor strain	Plasmid	Antibiotic added [a]		Number of transformants/ml [b]	
				Direct selection	Two-step selection
A)					
IA510	pC194	Cm	5	4×10^3	1.6×10^4
GSY1104	pE194	Em	30	4×10^3	4.7×10^3
	pE194	Em	0.2	3.4×10^3	2.9×10^3
	pBC16	Tc	15	5.4×10^3	6.3×10^3
GSY1035	pUB110	Km	30	3.1×10^5	1.4×10^5
GSY1277-3	pUB110	Km	30	9.2×10^4	7.8×10^4
GSY1104	pTV1	Em	0.2	1.6×10^2	8×10^2
		Cm	5	4.5×10^2	8×10^2
GSY1104	pPL608	Km	30	1.1×10^6	2×10^5
		Cm	5	4.8×10^5	2×10^5
GSY1104	pMK3	Neo	30	4.5×10^4	–
B)					
GSY1104	pPL608	Neo	30	1.6×10^5	2×10^5
		Neo	30	3.5×10^4 [d]	3×10^4 [d]
GSY1104 [c]	pPL608	Neo	30	5×10^4	7×10^4

[a] Antibiotic concentrations included in DM3 regeneration medium were in μg/ml.

[b] 10^8 protoplasts were transformed with nearly 100 ng of DNA in each experiment as indicated in MATERIALS AND METHODS. Dilutions were spread on DM3 media containing antibiotic (direct selection) or not (two-step selection). Approximately 10% of the protoplasts gave colonies on this last medium.

[c] Protoplasts of GSY1104 in SMM were kept frozen at −70 °C for several weeks. After rapid thawing at room temperature 0.5 ml were transformed and dilutions plated on regeneration DM3 media as indicated.

[d] A one-tenth dilution of the transformation mixture was kept frozen at −70 °C. After several days, it was thawed and dilutions spread on the same regeneration media.

eliminated. It is noteworthy that the minimal antibiotic concentration has to be carefully determined, depending on the antibiotic (see above for erythromycin) and regeneration medium used: 5 μg/ml of chloramphenicol allows expression on DM3, while this concentration has to be reduced to 1 or 2 μg/ml with mR2 medium. DM3, R or mR2 regeneration media gave similar efficiencies in transformation, but 1, 2 or 3 days of incubation were needed respectively. Utilization of other recently introduced media [18] which allow the recovery of bacilli after 17 h of incubation, results in a much lower regeneration efficiency (one hundredth to one thousandth) and thus in lower counts of transformants, a parameter which needs to be optimized if recombinant molecules are to be introduced.

DNA preparations from pMK3 (a bifunctional vector useful for cloning in *B. subtilis*) gave lower counts at the saturating concentration of plasmid DNA (which in our conditions is attained with nearly 200 ng), e.g. 4.10^4 Km^R transformants were obtained with this plasmid compared to 1.10^6 Km^R transformants with plasmid pPL608. This DNA preparation was obtained from *E. coli* cultures and restriction mechanisms could probably account for this reduction. However, saturation in this system depends on the concentration of DNA and that of protoplasts since ten times more transformants were obtained if the input number of protoplasts was increased by the same factor (3×10^7 Km^R transformants were obtained with 500 ng of DNA from pPL 608 and 2.10^9 protoplasts instead of 3.4×10^6 transformants if 2×10^6 protoplasts were transformed).

The ability of religated DNA to transform protoplasts with this procedure was also tested since competent cells were shown to fail as receptors with this DNA preparations [19,20]. With DNA from pPL608 digested and religated the number of transformants was 1.5×10^5, one third of the value obtained with the untreated plasmid (4.5×10^5) while the unligated control yielded less than 10 transformants in these experiments. These values are compatible with the use of this technique in cloning experiments.

Considering the practical aspects of this system, it was of interest to have a method to keep protoplast preparations for certain periods of time to render faster and easier their use. Freezing and storing of protoplast suspensions in SMM at −70 °C did not alter their viability significantly nor their transformability (Table 2B); in fact, dilutions of the transformation mixture have also been stored without a significant loss in recovery.

The application of this technique should also be amenable to Rec^- genetic backgrounds in spite of their low viability and regeneration efficiency. To overcome these problems the effect of several osmo-protective agents were assayed. The addition of L-proline, L-glutamic acid and betaine was in-

Table 3

Effect of osmoprotectants in regeneration and preservation

Expt.	Strain	Addition to SMM	Number of regenerants [a]	Number of regenerants [a] after storage at −70 °C
1)	GSY1104	none	4.5×10^{6}	2.8×10^{6}
		L-proline	2×10^{7}	1.2×10^{7}
		betaine	2.6×10^{7}	3×10^{6}
2)	IA510	none	2.6×10^{5}	1.3×10^{5}
		L-proline	5.2×10^{5}	3×10^{5}
		L-glutamate	1.5×10^{7}	8.7×10^{6}
3)	IA510	none	3.4×10^{7} [b]	1.6×10^{7} [b]
		betaine	3.8×10^{7} [b]	1×10^{7} [b]

Protoplasts were prepared as indicated in MATERIALS AND METHODS in SMM-lisozyme buffer. Addition of L-proline, betaine or L-glutamate acid was 0.1% in SMM.

[a] protoplast concentration was $4\cdot10^{8}$/ml; regeneration medium was DM3.

[b] regeneration medium was mR with reduced concentration of sodium succinate (0.25 M), addition of L-glutamate 0.1%,and auxotrophic requirements.

vestigated. Their presence during the protoplast formation and storage steps, increased protoplast viability and regeneration efficiency (Table 3), although the incubation time needed to convert bacilli to protoplasts is longer in these media. In spite of being more stable (and probably due to this reason), IA510 protoplasts stored in this way were less transformable. However, this unwanted effect could be overcome by raising the PEG concentration to 40% in the transformation mixtures.

It was also our experience that Rec^{-} protoplasts regenerated much better in minimal mR2. Addition of 0.1% L-glutamate in this medium restored their regeneration to values comparable to those of the Rec^{+} protoplasts (Table 3). It is noteworthy that effectors increasing the recovery of the bacillary form have also been shown to affect the morphological behavior of *rod-4* mutants which change from round to rod-shaped cells [21].

In conclusion, the improvements introduced to the protoplast transformation technique allow to easily and efficiently apply it to a variety of genetic manipulations in *B. subtilis,* including molecular cloning experiments.

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REFERENCES

[1] Chang, S. and Cohen, S.N. (1979) Mol. Gen. Genet. 168, 111–115.
[2] Gryczan, T., Contente, S. and Dubnau, D. (1980) Mol. Gen. Genet. 177, 459–467.
[3] Rudolph, C.F., Schmidt, B.J. and Saunders, C.W. (1986) J. Bacteriol. 165, 1015–1018.
[4] Schaeffer, P., Cami, B. and Hotchkiss, R.D. (1976) Proc. Natl. Acad. Sci. USA 73, 2151–2155.
[5] Sanchez-Rivas, C. (1982) Mol. Gen. Genet. 185, 329–333.
[6] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523.
[7] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY.
[8] Levy-Meyrueis, C., Sanchez-Rivas, C. and Schaeffer, P. (1980) Compt. Rend. Acad. Sci. 291, 67–70 ser.D.
[9] Sullivan, M.A., Yasbin, R. and Young, F. (1984) Gene 29, 21–26.
[10] Ostroff, G.R., Pène, J.J. (1983) J. Bacteriol. 156, 934.
[11] Iordanescu, S., Surdeanu, M., Della Latta, P. and Novick, R. (1978) Plasmid 1, 468–479.
[12] Bernhard, K., Schrempf, H. and Goebel, W. (1978) J. Bacteriol. 133, 897–903.
[13] Keggins, K., Lovett, P., Duvall, E. (1978) Proc. Natl. Acad. Sci. USA 75, 1423–1427.
[14] Youngman, P., Perkings, J.B. and Losick, R. (1983) Proc. Natl. Acad. Sci. USA 80, 2305.
[15] Williams, D.M., Duvall, E.J. and Lovett, P.S. (1981) J. Bacteriol. 146, 199–206.
[16] Sanchez-Rivas, C. (1988) Annales Institut Pasteur. In press.
[17] Kopec, L.K., Yasbin, R.E. and Marrero, R. (1985) J. Bacteriol. 164, 1283–1287.
[18] Puyet, A., Sandoval, H., Lopez, P., Aguilar, A., Martin, J.F. and Espinosa, M. (1987) FEMS Microbiol. Letters 40, 1–5.
[19] Contente, S. and Dubnau, D. (1979) Plasmid 2, 555–571.
[20] Rapoport, G., Klier, A., Billant, A., Fargette, F. and Dedonder, R. (1979) Mol. Gen. Genet. 176, 239–245.
[21] Rogers, H.J. and McConnell, M. (1970) J. Gen. Microbiol. 61, 173–181.

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The effect of thiols and the Ca^{2+} ionophore A23817 on growth and antibiotic production in *Cephalosporium acremonium*

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1. SUMMARY

Yeast-like blastosporic growth, lowered levels of isopenicillin N synthetase and deacetoxycephalosporin C synthetase/hydroxylase, and lowered antibiotic production were induced in *Cephalosporium acremonium* strain C.O. 728 by inclusion of cysteine or glutathione in the growth medium. These effects were similar to those observed under normal conditions with strain M 8650/4, which had higher levels of glutathione reductase and protein disulphide reductase than strain C.O. 728. The addition of cytochalasins A and B slightly increased yeast-like growth in C.O. 728, although levels of biosynthetic enzyme activity and antibiotic secretion were not significantly affected. The Ca^{2+} ionophore A23187 inhibited C.O. 728 growth, slowed penicillin secretion into the medium, and totally inhibited cephalosporin secretion. These results show that maintenance of Ca^{2+} levels and the absence of thiols are important for antibiotic production in *Cephalosporium acremonium.*

2. INTRODUCTION

Fungi grow by means of hyphae which elongate at the apices [1]. During growth, new wall deposition occurs at the apices [2,3], and hydrolytic enzymes are probably secreted via the apical tips [1]. A number of factors may alter the apical tip wall structure, and so effect cell function and morphology.

In some forms of *Cephalosporium acremonium* (*Acremonium chrysogenum*), large numbers of budding yeast-like blastospores may appear in submerged culture [4,5]. This change in morphology from normal hyphal growth has been produced by increasing methionine levels in the medium [6], with a corresponding decrease in cephalosporin production. The ability to synthesise cephalosporin C correlated with the ability to form thick hyphal fragments [7].

Disturbance of the cytoskeleton can dramatically influence tip wall deposition [8,9]. Transhyphal currents of ions, notably Ca^{2+}, appear necessary for the proper functioning of processes based on the cytoskeleton [10]. In pollen tubes, treatment with cytochalasin B led to inhibition of

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cytoplasmic streaming, tip growth and germination [11], while treatment with ionophores led to disruption of both the calcium gradient and the cytoskeleton, together with cessation of wall secretion and growth [12,13].

This paper reports the effects of thiols, cytochalasins and the Ca^{2+} ionophore A 23817 on growth and antibiotic production in *C. acremonium*.

3. MATERIALS AND METHODS

3.1. Materials

Ionophore A23187, phorbol ester, amiloride and cytochalasins A and B were obtained from Sigma Chemical Co., London. All other materials were as described previously [14,15].

3.2. Growth of organism

Cephalosporium acremonium C.O. 728 and M8650/4 (kindly provided by Eli Lilly and Co.) were grown in chemically defined medium as described previously [14,15]. The cytochalasins and the ionophore were made up in 1% dimethylsulphoxide (DMSO). Control cultures containing 1% DMSO were compared with cultures grown without DMSO. Cells were photographed using Zeiss and Olympus photo-microscopes. Percentages of yeast and mycelial cell-types were estimated microscopically. Antibiotic production was determined by bioassay as described previously [15].

3.3. Preparation of cell-free extracts

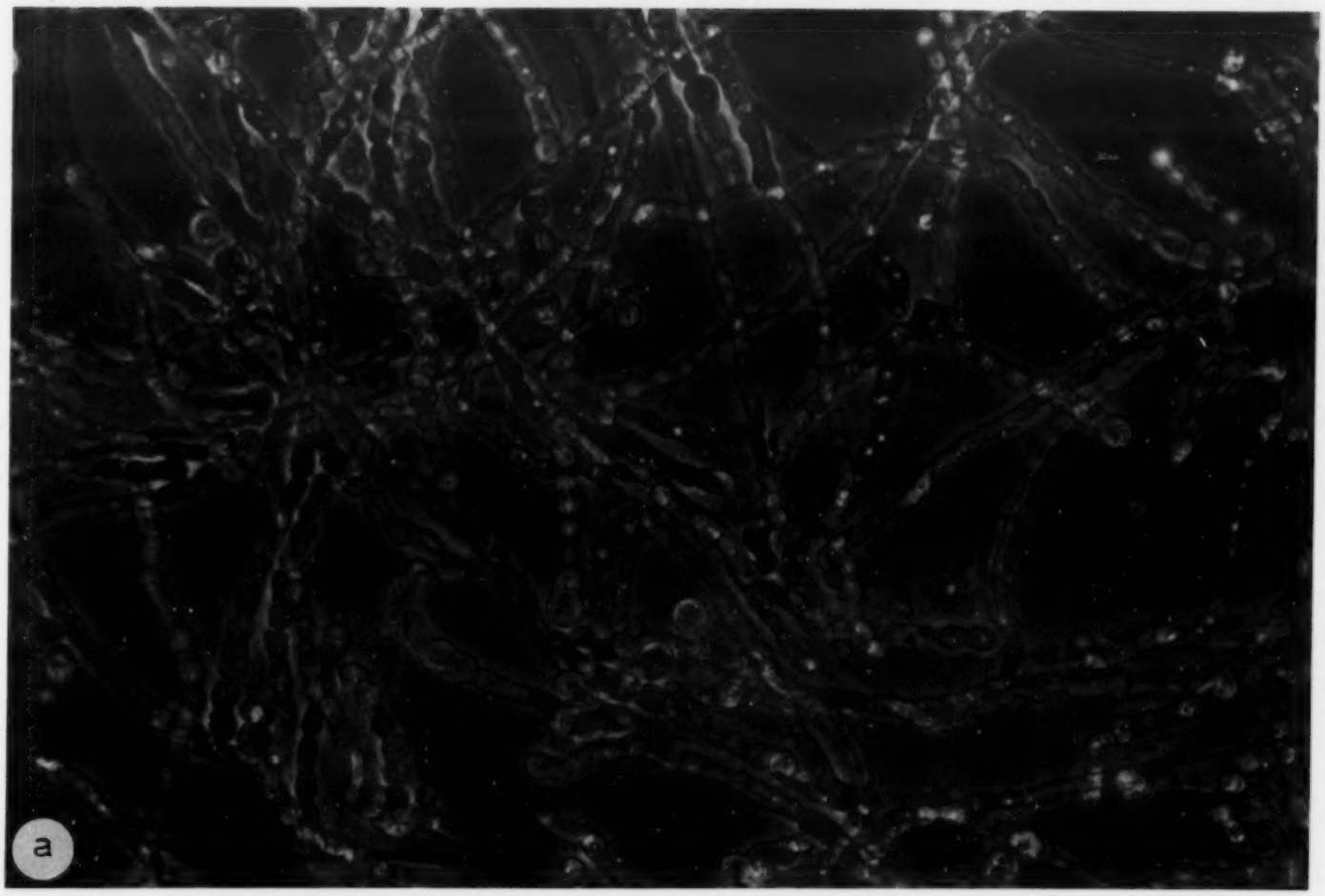
Fungal cells (20 g) suspended in 50 mM Tris/HCl buffer (50 ml), pH 7.7, containing 1% Triton X-100 were subjected to sonication at 4°C using an MSE soniprobe for 3 min bursts with 3 min rests in between, for a total of 15 min with C.O. 728 and 30 min with M8650/4. These conditions were arrived at after microscopic examination and biosynthetic enzyme assays. Cell debris was removed by centrifugation at $12\,000 \times g$ for 20 min, and the supernatant treated as described previously [14,15].

3.4. Enzyme assays

Isopenicillin N synthetase and deacetoxycephalosporin C synthetase/hydroxylase were assayed as described previously [14–16]. Glutathione reductase was assayed by incubation of extract (300 μl), oxidised glutathione (GSSG) (1 mM) and NADPH (0.1 mM) in 50 mM Tris/HCl buffer, pH 7.4 in a spectrophotometer cell at 26°C, monitoring the absorbancy change spectrophotometrically at 340 nm against a blank made up as above but without GSSG. Protein disulphide reductase was assayed as described in [17]. 1 I.U. of enzyme activity is defined as that amount catalysing the utilisation of 1 μmol of substrate/min under standard conditions.

4. RESULTS AND DISCUSSION

In preliminary experiments, it was found that *C. acremonium* C.O. 728 produced mycelial growth, without any significant yeast-like blastospores, over the medium pH range 6.5–7.8, medium glucose concentrations of 1.35%–5.4%, and ages of inocula from 58 h–82 h (Fig. 1a). In contrast, *C. acremonium* M8650/4 produced large numbers of blastospores under these conditions (about 80% of cells were yeast-like) (Fig. 1b), and only when oxygenation was high (using 80 ml culture volume in shake flasks rather than the usual 100–120 ml) was there significant mycelial growth (about 80% mycelial cells). Typical biosynthetic enzyme specific activities obtained from the C.O. 728 mycelial growth were 1.0×10^{-3} I.U./mg for isopenicillin N synthetase and 5.0×10^{-4} I.U./mg for deacetoxycephalosporin C synthetase/hydroxylase. The yeast-like form of the M8650/4 strain produced over ten-fold lower levels of isopenicillin N synthetase (5.0×10^{-5}) and deacetoxycephalosporin C synthetase/hydroxylase (2.0×10^{-5}) specific activities than the mycelial form. In accord with the findings of Nash and Huber [7], there was a significant reduction in penicillin and cephalosporin production into the medium in cultures containing large amounts of yeast-like cells, from about 0.1 mg/ml of penicillin N and cephalosporin C after 100 h with mycelial cells to about 0.005 mg/ml with yeast-like cells. When strain M8650/4 was induced to grow in the mycelial form, levels of biosynthetic enzyme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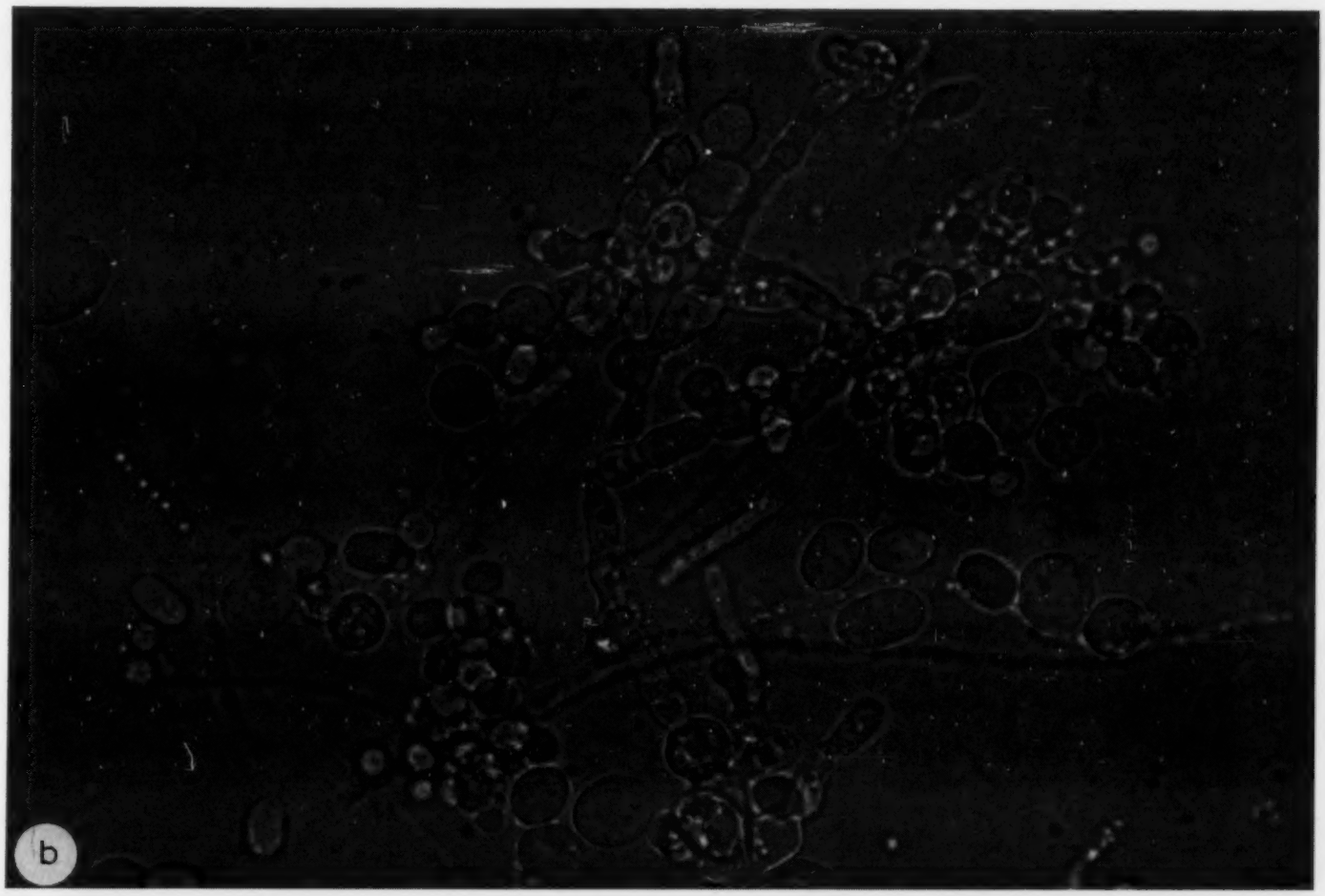
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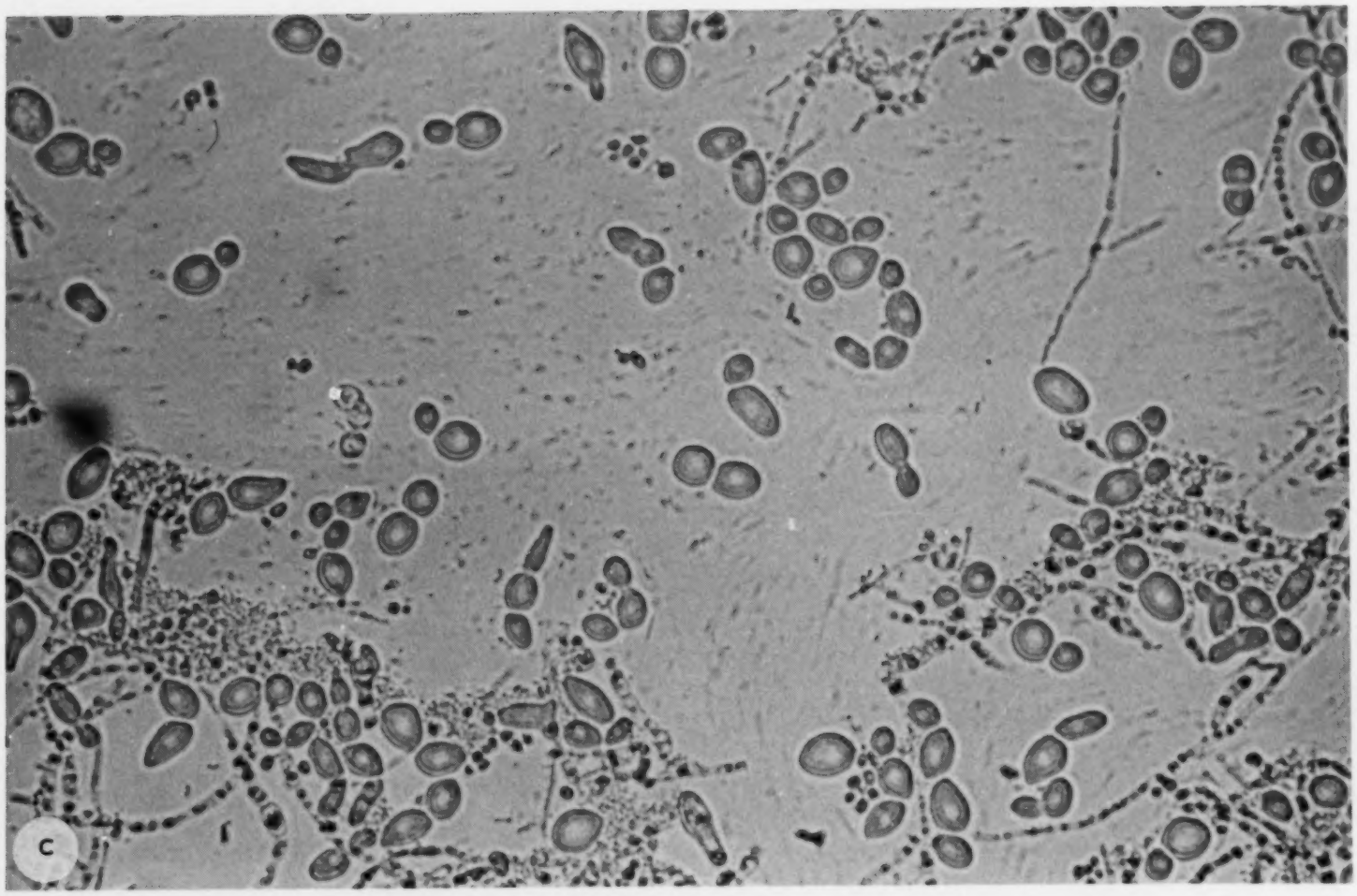
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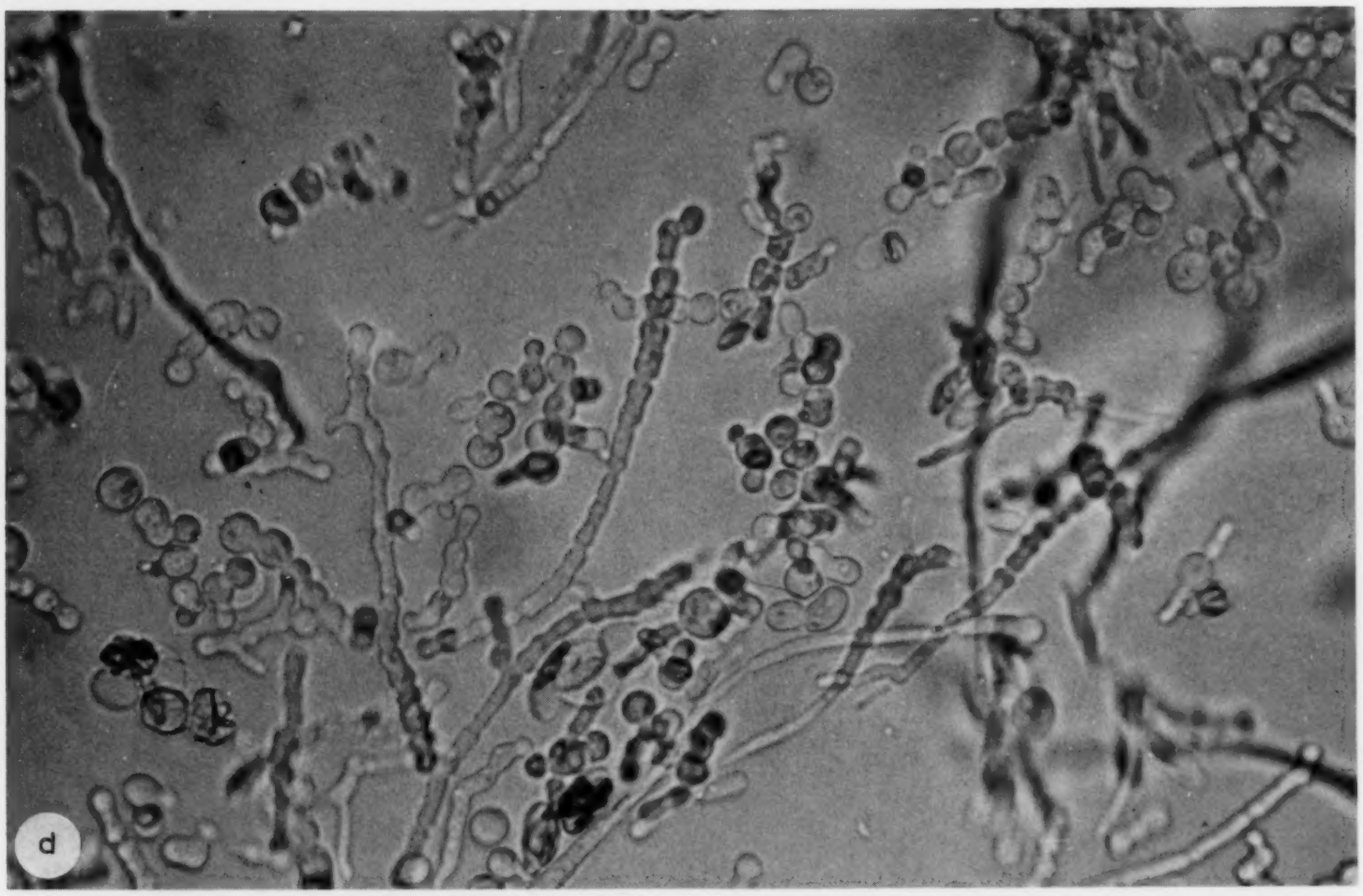
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Table 1

Specific activities of GSSG reductase and protein disulphide reductase

Cephalosporium acremonium strain	GSSG reductase	Protein S-S reductase
C.O. 728	0.86	not detectable
M8650/4	6.29	2.48

For details see text. Units are I.U./mg$\times 10^5$.

activities and antibiotic production were similar to the mycelial C.O. 728 strain.

The addition of cysteine (10 mM) and reduced glutathione (GSH, 10 mM) to the medium with C.O. 728 resulted in almost complete conversion to blastospores (about 90% yeast-like cells), as illustrated in Fig. 1c, with activity levels of biosynthetic enzymes and antibiotic production lowered to those of the yeast-like form of M8650/4.

Table 1 shows the levels of GSSG reductase and protein disulphide reductase in extracts of C.O. 728 and M8650/4. In the mycelial C.O. 728 there were considerably lower levels of both reductases than in M8650/4, which was the strain prone to develop blastospores as described by Queener and Ellis (1975) [5]. This may reflect a more rigid cell wall in C.O. 728 than in M8650/4. If disulphide crosslinked protein is present in the cell wall of *C. acremonium,* and is necessary for maintenance of normal cell morphology, as it is in the model yeast *Saccharomyces cervisiae* [1], then reduction of the disulphide crosslinks may lead to loss of hyphal morphology and increased 'rounding' of cells to form the budding yeast-like cells. The reductive environment of cysteine and GSH would promote disulphide bond reduction, while the higher levels of the reductases found in M8650/4 would make that strain more prone to disulphide bond breakage than strain C.O. 728. In accord with this, an oxidative environment (e.g.

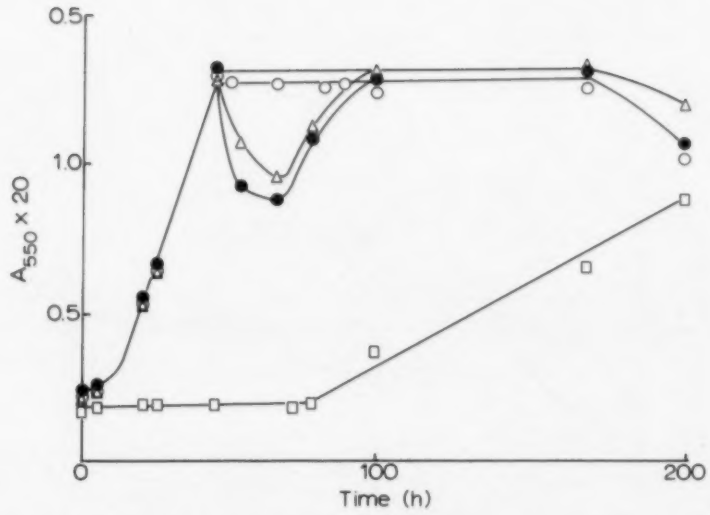

Fig. 2. Growth profiles of *Cephalosporium acremonium* C.O. 728. ○, control + DMSO (1%); △, medium + cytochalasin A (11 μM); ●, medium + cytochalasin B (11 μM); □, medium + ionophore A 23187 (0.05 mM). Growth profile of control + DMSO (1%) was identical to control without any additives. The addition of Ca^{2+} (0.05 mM) or cAMP (1–7 mM) to the medium containing the ionophore produced no significant modifications to the growth profile. For details see the text.

increased oxygenation) would conserve the disulphide-bonded protein, as was found experimentally with M8650/4.

Fig. 2 shows the effect of the actin-binding drugs cytochalasin A and B and the calcium ionophore A 23187 on the growth of *C. acremonium* C.O. 728. The presence of DMSO in the medium did not change the growth profile, the cellular morphology, the levels of the biosynthetic enzymes or antibiotic secretion into the medium. Growth in media containing cytochalasins A and B showed initial growth profiles and morphologies similar to the controls until about 50 h after inoculation, when the A_{550} dropped and a significant number of yeast-like blastospores developed (about 40% yeast-like cells) (Fig. 1d). However, the percentage of blastospores did not increase as

Fig. 1. Morphology of *Cephalosporium acremonium* strains C.O. 728 and M8650/4 under different culture conditions. For details see the text. (a) C.O. 728 under control conditions, 50 h after inoculation. No changes in morphology were seen up to 160 h after inoculation, or if 1% DMSO was included in culture media. ×400, phase contrast. (b) M8650/4 under control conditions, 74 h after inoculation. No significant changes in morphology were seen between 66 h and 110 h after inoculation. ×400, oil immersion, yellow filter. (c) C.O. 728, grown as in (a), but with the addition of cysteine (10 mM) and GSH (10 mM) to the medium. 74 h after inoculation. ×400, yellow filter. (d) C.O. 728, grown as in (a), but with the addition of cytochalasin A (11 μM) to the medium. 56 h after inoculation. Cultures containing cytochalasin B showed similar morphology from 45 h after inoculation. ×400.

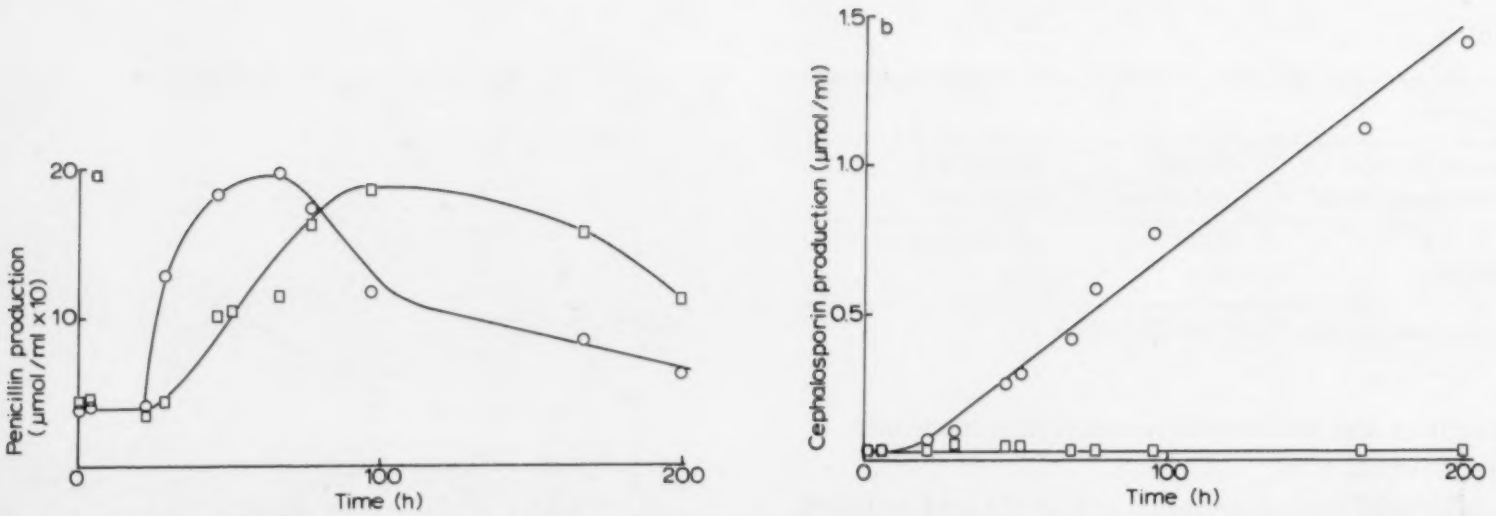

Fig. 3. Antibiotic production in the culture medium of *Cephalosporium acremonium* C.O. 728. For details see te text. (a) Penicillin production. ○, control; □, medium + ionophore A23187. There were no significant differences in penicillin production between the control and in cultures containing DMSO (1%) or cytochalasin A or B (11 μM). (b) Cephalosporin production. ○, control; □, medium + ionophore A23187. There were no significant differences in cephalosporin production between the control and in cultures containing DMSO (1%) or cytochalasin A or B (11 μM).

culturing continued, but rather decreased to about 20% of cells after 120 h. This was in contrast to cultures of M8650/4 or C.O. 728 with glutathione and cysteine, where the yeast-like cells completely dominated at the end of stationary phase.

Growth in medium containing the ionophore was significantly slower than the controls, with thin hyphal growth similar to that seen just after inoculation. Again, a number of yeast-like blastospores were present (about 60% yeast-like cells). Activities of the antibiotic biosynthetic enzymes corresponded to control cultures at similar cell densities.

Fig. 3 shows the antibiotic production in the cultures of strain C.O. 728. Cytochalasins A and B did not significantly effect biosynthetic enzyme activity, penicillin or cephalosporin production from the fungus, despite the yeast-like cells apparent at the start of stationary phase. However, the Ca^{2+} ionophore A 23817 slowed penicillin secretion, with a peak at 100 h after inoculation rather than at the normal 70 h. Cephalosporin secretion was completely inhibited. The addition of cAMP (1–7 mM) or Ca^{2+} (0.05 mM) to the medium containing ionophore produced no significant alterations in these results. The addition of the phorbol ester 12-O-tetradecanoylphorbol-13 acetate (TPA) (0.8 μM) or amiloride (0.5 mM), an inhibitor of Na^+/H^+ counter-transport, produced no major effect on growth or antibiotic production. The lack of effect of the phorbol ester or amiloride suggests that diacylglycerol or Na^+/H^+ exchange mechanisms were probably not of major importance in antibiotic secretion.

These results show that drugs binding to actin caused some modifications to the morphology of *C. acremonium* by increasing the number of yeast-like cells. However, this morphological change did not interfere with penicillin or cephalosporin biosynthesis or secretion. The formation of yeast-like blastospores was very marked when cells were grown in a reductive environment with GSH and cysteine. When blastosporic cells dominated the culture, lower amounts of penicillin N, cephalosporin C, isopenicillin N synthetase and deacetoxycephalosporin C synthetase/hydroxylase were produced than in normal mycelial cells. Thus conversion of mycelial into almost totally yeast-like cells resulted in a 'switching-off' of the penicillin and cephalosporin biosynthetic pathway, at least at the level of isopenicillin N synthetase and deacetoxycephalosporin C synthetase/hydroxylase. The Ca^{2+} ionophore A23187 delayed cell growth, lowered biosynthetic enzyme activities and penicillin secretion, and completely inhibited cephalosporin secretion. Maintenance of Ca^{2+} levels does appear to be crucial both for growth and antibiotic secretion, particularly in the

case of cephalosporins. Indeed, the differential response of penicillin and cephalosporin secretion to the ionophore may reflect subtle differences in secretory mechanisms for these two antibiotics.

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REFERENCES

[1] Wessels, J.G.H. (1986) Int. Rev. Cytol. 104, 37–79.
[2] Bartnicki-Garcia, S. (1973) Symp. Soc. Gen. Microbiol. 23, 245–267.
[3] Wessels, J.G.H., Sietsma, J.H. and Sonnenberg, A.S.M. (1983) J. Gen. Microbiol. 129, 1607–1616.
[4] Jicinska, E. (1974) Folia Microbiol 19, 1–4.
[5] Queener, S.W. and Ellis, L.F. (1975) Can. J. Microbiol. 21, 1981–1996.
[6] Caltrider, P.G., Huber, F.M. and Day, L.E. (1968) Appl. Microbiol. 16, 1913–1918.
[7] Nash, C.H. and Huber, F.M. (1971) Appl. Microbiol 22, 6–10.
[8] Allen, E.D., Aiuto, R. and Sussman, A.S. (1978) Protoplasma 102, 63–75.
[9] Herr, F.B. and Health, M.C. (1982) Exp. Mycol. 6, 15–24.
[10] Reissig, J.L. and Kinney, S.G. (1983) J. Bacteriol. 154, 1397–1402.
[11] Franke, W.W., Herth, W., VanDer Woude, W.J. and Morre, D.J. (1972) Planta 105, 317–341.
[12] Reiss, H.-D. and Herth, W. (1979) Planta 145, 225–232.
[13] Reiss, H.-D. and Herth, W. (1982) Planta 156, 218–225.
[14] Pang, C.-P., Chakravarti, B., Adlington, R.M., Ting, H.-H., White, R.L., Jayatilake, G.S., Baldwin, J.E. and Abraham, E.P. (1984) Biochem. J. 222, 789–795.
[15] Baldwin, J.E., Adlington, R.M., Coates, J.B., Crabbe, M.J.C., Crouch, N.P., Keeping, J.W., Knight, G.C., Schofield, C.J., Ting, H.-H., Vallejo, C.A., Thorniley, M. and Abraham, E.P. (1987) Biochem. J. 245, 831–841.
[16] Baldwin, J.E. and Crabbe, M.J.C. (1987) FEBS Lett. 214, 357–361.
[17] Morin, J.E., Carmichael, D.F. and Dixon, J.E. (1978) Arch. Biochem. Biophys. 189, 354–363.

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The properties and structure of the membrane ATPase from *Vibrio alginolyticus*

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Key words: *Vibrio alginolyticus*; Adenosine triphosphatase; Cell membrane

1. SUMMARY

Membrane ATPase of marine alkali-tolerant bacterium *Vibrio alginolyticus* has been studied. The ATPase was inhibited by diethylstilbestrol, DCCD, NBD-Cl and sodium azide, but it was insensitive to vanadate. The ATPase was solubilized by EDTA-treatment and partially purified by ion exchange chromatography. Two major polypeptides with molecular weights of 55 kDa and 58 kDa were found in this preparation. The molecular weight of the soluble ATPase was estimated to be 360 kDa. These data indicate, that the ATPase of *Vibrio alginolyticus* is likely to belong to F_0–F_1 type.

2. INTRODUCTION

Several energy-consuming processes in cells of the marine alkali-tolerant bacterium *Vibrio alginolyticus* were reported to depend on the electrochemical gradient of Na^+-ions ($\Delta\mu Na^+$), i.e., the transport of metabolites [1], rotation of the flagella [2] and ATP-synthesis [3]. Na^+-dependent NADH-oxidase, functioning as an electrogenic Na^+-pump has been found in the cell membrane [4]. Recently, $\Delta\mu H^+$ generation in the respiratory chain and ATP-dependent proton pumping has been demonstrated in membrane vesicles from *V. alginolyticus* [5]. These results indicate that both $\Delta\mu Na^+$ and $\Delta\mu H^+$ are involved in the bioenergetic mechanisms of this bacterium. The present work deals with the membrane ATPase of *V. alginolyticus*.

3. MATERIALS AND METHODS

3.1. Cultivation of bacteria and preparation of membrane vesicles

V. Alginolyticus, strain 138-2, was a gift of H. Tokuda (Chiba University, Japan). Bacteria were grown anaerobically in a medium, containing 0.5 m NaCl, 10 mM KCl, 5 mM $MgSO_4$, 15 mM $(NH_4)_2SO_4$, 2 mM KH_2PO_4, 50 mM Tris-HCl, pH 8.5, 0.8% glucose, and 0.5% tryptic soy broth. Spheroplasts, obtained by penicillin treatment were washed twice with an isotonic solution and resuspended in a medium, containing 30 mM $MgSO_4$, 10 mM Na_2SO_4, 25 mM HEPES-KOH, pH 7.6, 1 mM phenylmethylsulfonylfluoride, and 30% ethyleneglycol. The suspension was cooled to −15°C and sonicated. Debris were removed by

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centrifugation (10 min at $25000 \times g$) and the vesicles pelleted (1 h at $50000 \times g$, $-5°C$). Vesicles were washed with the same solution and stored at $-40°C$.

3.2. Analytic procedures

ATPase was assayed either by liberation of inorganic phosphate by the method of Lin and Morales [6] or by ADP-production in enzyme-coupled regenerating system with piruvate kinase and lactate dehydrogenase [7] at 28°C. The assay medium routinely contained 100 mM Tris-MES, pH 7.6, 7 mM $MgSO_4$ and 2 mM ATP.

3.3. Chromatography techniques

The extract, containing soluble ATPase, was applied to a DEAE-Sephacel column (2.8×15 cm) in a buffer, containing 10 mM Tris-MES, pH 8.0, 5 mM ATP, 20% ethyleneglycol, 0.1 mM phenylmethylsulfonylfluoride. The protein was eluted with this buffer, supplemented with NaCl (linear gradient 0–0.5 M). Gel filtration was performed on a Toyo-pearl HW-60 column (2.8×100 cm) equilibrated with the same buffer.

3.4. Chemicals

Tris, MES, HEPES, ATP were from Sigma; DCCD, diethylstilbestrol, NBD-chloride from Serva; DEAE-Sephacel from Pharmacia; Toyo-pearl from Toyo-Soda. Other chemicals were of analytical grade.

4. RESULTS

4.1. ATP-hydrolysing activity of V. alginolyticus *membranes*

The rate of P_i production from ATP in membrane vesicles was 0.2–0.26 μmol/min mg of protein and the rate of AMP hydrolysis varied from 0.05 to 0.15 μmol/min mg protein. AMP-hydrolysis was stimulated by Cl^+-ions 4- to 5-fold with a half maximal effect at 5 mM. These data are in good agreement with the results of Hayashi et al. [8], who found Cl^--dependent nucleotidase in subcellular preparations from *V. alginolyticus*. We found that AMP hydrolysis had pH-optimum at 8.5 and was inhibited by fluoride and *ortho*-vanadate (Table 1). Besides AMP and GMP, Cl^--dependent nucleotidase hydrolysed ADP, ATP, GTP, ITP, while pyrophosphate and cyclic AMP were not hydrolysed.

TABLE 1

Inhibitor sensitivity of the ATPase and nucleotidase

Inhibitor	Concentration	Percent of inhibition	
		ATP-ase	Nucleotidase
DCCD	100 μM	75	6
NBD-Cl	100 μM	77	5
Sodium azide	1 mM	75	n.d.
Diethylstilbestrol	20 μM	59	n.d.
	100 μM	91	n.d.
N-ethylmaleimide	1 mM	29	n.d.
ortho-vanadate	200 μM	18	45
	1 mM	32	75
Sodium fluoride	10 mM	10	79
DCCD + *o*-vanadate	100 μM + + 200 μM	78	n.d.

ATPase activity was assayed with enzyme coupled ATP-regenerating system at pH 7.6, nucleotidase activity was measured by P_i liberation with AMP as a substrate. *o*-vanadate, fluoride, azide or diethylstilbestrol were added to the assay medium. In the experiments with DCCD, NBD-Cl and *N*-ethylmaleimide, the membrane vesicles were preincubated with the inhibitor and then diluted with the assay medium 10- to 20-fold.

The inhibitors of F_0–F_1-type ATPases, DCCD and NBD-chloride, partially blocked the ATP hydrolysis (approximately by 40%) but did not affect the rate of AMP hydrolysis. These data suggest that ATP was also hydrolysed by another enzyme – ATPase. ADP-production, measured in the enzyme-coupled regenerating system, was neither stimulated by chloride nor inhibited by AMP which must have competitively inhibited the ATP-hydrolysing activity of nucleotidase, Thus, only ATPase was measured by this method, while the nucleotidase activity was not revealed. The ATPase activity was 0.15–0.20 μmol/min mg protein.

4.2. Properties of the membrane ATPase

The ATPase activity had strong pH-dependence with an optimum at pH 7.6. The ATPase reaction obeyed the Michaelis-Menten equation, and K_m for ATP was found to be 0.25 mM (the concentration of Mg^{2+} was 7 mM). The excess of

Mg^{2+} up to 10 mM had no effect on ATPase activity. The ATPase showed a clear preference for purine nucleotides and Mg^{2+} as substrates (Table 2).

The characteristic inhibitors of F_0–F_1 ATPases DCCD, NBD-chloride and sodium azide suppressed the activity of *V. alginolyticus* ATPase. Diethylstilbestrol also proved to be a potent inhibitor of the ATPase. Fluoride and *ortho*-vanadate had no effect on the ATPase activity (Table 1). The ATPase was not significantly activated by trypsin, detergents or dithiotreitol. Methanol (10% in the assay medium) activated the ATPase by 60%.

We have not found any measurable effect of Na^+-ions in the range of 0.03–50 mM on the ATPase activity. Na^+ had no effect on the ATPase both at the optimal pH 7.6 and higher pH up to 9.0. Varying the conditions of bacterial cultivation (pH, oxygen supply) has not revealed Na^+-dependent ATPase either.

4.3. The soluble ATPase

ATPase activity could be completely detached from membranes by treatment with EDTA (3 mM) in the presence of 5 mM ATP and 30% ethyleneglycol. Specific activity of the extract was 0.3–0.4 μmol/min mg protein. The soluble ATPase retained its sensitivity to NBD-chloride and sodium azide. Diethylstilbestrol, however, had no effect on the soluble ATPase, and its sensitivity to DCCD also considerably decreased.

The soluble ATPase was purified approximately tenfold by anion exchange chromatography. SDS-electrophoresis, performed according to Laemmli [9], revealed two major types of polypeptides with a molecular weight of 55 kDa and 58 kDa in this preparation (Fig. 1). The molecular weight of the soluble ATPase, as estimated by gel filtration, was approximately 360 kDa.

TABLE 2

Substrate specificity of the ATPase

Substrate	Activity %	Substrate	Activity %
Mg-ATP	100	Mg-ATP	100
Mg-ITP	71	Ca-ATP	68
Mg-GTP	29	Mn-ATP	14
Mg-UTP	10	Zn-ATP	2
Mg-CTP	5		

The values in the table are the relative DCCD-sensitive activities, measured by P_i liberation. The DCCD sensitive activity with Mg-ATP as substrate was 0.14 μmol/min mg protein.

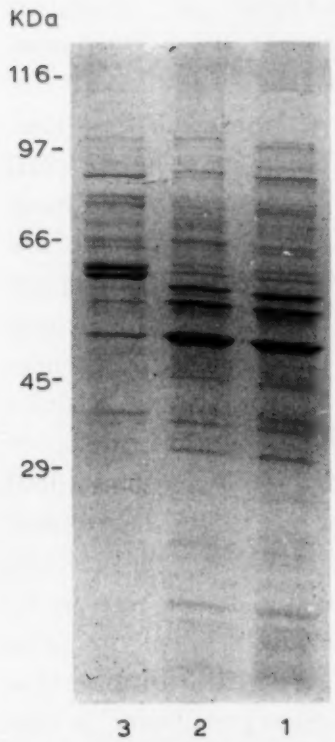

Fig. 1. Polypeptide composition of the membrane vesicles (1), EDTA-extract (2) and partially purified ATPase (3) as revealed by SDS-electrophoresis in Laemmli's system. 100 μg protein was applied to each lane. Positions of molecular weight markers are shown on the left.

5. DISCUSSION

The membrane ATPase of *V. alginolyticus* was sensitive to characteristic inhibitors of F_0–F_1-type ATPase DCCD, NBD-chloride and sodium azide. The decrease of the ATPase sensitivity to DES and DCCD after solubilization by EDTA-treatment may evidence for its two-component structure, similar to that of F_0–F_1 ATPases. Finally, two major polypeptides, found in the partially purified preparation of the soluble ATPase resemble α- and β- subunits of typical F_1–ATPases. Thus, the ATPase of *V. alginolyticus* is probably of F_0–F_1 type.

Both the membrane ATPase and oxidative phosphorylation in the cells of *V. alginolyticus* were inhibited by DES and DCCD [10]. ATP-dependent generation of electric potential in subcellular vesicles from *V. alginolyticus* was also blocked by DCCD [11]. These data suggest that the above described ATPase catalysed the electrogenic ion transport and ATP synthesis in cells of *V. alginolyticus*. In our experiments ATPase activity was not enhanced by Na^+. The ATP-dependent generation of electric potential in membrane vesicles from *V. alginolyticus* was also independent of Na^+-concentration [12]; moreover ATP-dependent proton transport was registered in these preparations [5]. However the ATP-synthesis in the cells of *V. alginolyticus* was reported to be driven by $\Delta\mu Na^+$ [3]. This discrepancy might have come as a result of experimental artifacts in the preparation of membrane vesicles. Another possibility is that the ATPase could transport H^+ as well as Na^+. The Na^+-transporting ATPase of F_0–F_1 type was recently isolated from *Propionigenium modestum* and was reported to transport protons as well [13]. Possibly, the ATPase of *V. alginolyticus* hydrolysed ATP at the same rate both for Na^+- and H^+-transport.

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REFERENCES

[1] Eddy, A. (1984) Curr. Top. Membr. Transp. 2, 250–348.
[2] Chernyak, B.V., Dibrov, P.A., Glagolev, A.N., Sherman, M.Yu. and Skulachev, V.P., (1983) FEBS Lett. 164, 36–42.
[3] Dibrov, P.A., Kostyrko, V.A., Lazarova, R.L., Skulachev, V.P. and Smirnova, I.A. (1986) Biochim. Biophys. Acta 250, 449–457.
[4] Tokuda, T. and Unemoto, H. (1981) Biochem. Biophys. Res. Comms. 102, 256–271.
[5] Smirnova, I.A., Vaghina, M.O., Kostyrko, V.A. (1988) 5th Soviet-Swiss Symp. Abstr. Riga, 110.
[6] Lin, T.C. and Morales, F. (1977) Anal. Biochem. 77, 10–18.
[7] Scharchmidt, B.F., Keeffe, E.B., Blunkenship, N.H. and Ockner, D.K. (1979) J. Lab. Clin. Med. 93, 2375–2382.
[8] Hayashi, M., Unemoto, T., Kozuka, Y. and Hayashi, M. (1970) Biochim. Biophys. Acta 220, 244–255.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Dibrov, P.A., Lazarova, R.L., Skulachev, V.P. and Verkhovskaya, M.L. (1986) Biochim. Biophys. Acta 250, 457–465.
[11] Smirnova, I.A., Vulfson, E.N. and Kostyrko, V.A. (1987) FEBS Lett. 214, 343–346.
[12] Dimroth, P. (1986) 17th FEBS Meet. Abstr., West Berlin, 66.

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Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation

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Key words: *Clostridium acetobutylicum*; Transformation; Cloning vector; Electroporation

1. SUMMARY

A method is presented for the introduction of plasmids into *Clostridium acetobutylicum* ATCC 8052 by electroporation. A plasmid shuttle vector, pMTL500E, which contains the erythromycin resistance gene and replication machinery of plasmid pAMβ1, was constructed and introduced into *C. acetobutylicum* by electroporation. The vector was then used to introduce a 2.2 kb *Cla*I/*Sph*I chromosomal fragment from *C. pasteurianum* into a leucine requiring mutant of *C. acetobutylicum*, SBA9, where complementation of auxotrophy was observed. Plasmid DNA indistinguishable from that introduced, on the basis of agarose gel electrophoresis, was observed in transformants containing either plasmid.

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2. INTRODUCTION

Recent research interest in the saccharolytic, solventogenic anaerobes has prompted the development of several techniques for their genetic manipulation (conjugal gene transfer and protoplast fusion in *Clostridium acetobutylicum*; [1–3], protoplast transformation in *C. saccharoperbutylacetonicum*, [4]), however, these techniques are either technically cumbersome, time consuming or limited in the number and type of plasmid vectors which they accommodate. As an alternative to these methods we have investigated the use of electroporation to mediate plasmid transformation of whole cells of *C. acetobutylicum* ATCC 8052. Electroporation has recently been used to introduce DNA into protoplasts and whole cells of several Gram-positive genera (*Streptomyces lividans* [5], *lactobacillus casei* [6], *Streptococcus lactis* and *S. thermophilus* [7,8]) as well as Gram-negative bacteria [9,10], yeast cells [11] and plant protoplasts [12]. The experiments below document the construction of a plasmid vector, pMTL500E,

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and its introduction into *C. acetobutylicum* by electroporation. Further the plasmid has been used to introduce a chromosomal fragment derived from *C. pasteurianum* into *C. acetobutylicum,* SBA9 (*leu*$^-$), where it complemented leucine auxotrophy.

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

Clostridium acetobutylicum ATCC 8052 and the leucine requiring mutant SBA9 were obtained from U.C.W. Aberystwyth. Plasmids were maintained in *Escherichia coli* strains JM83 (K12, *ara*, Δ(*lac-pro*)*rps*L, *thi* (φ80d*lac*I^{q}ZΔM15)) [13] and TG1 (K12, Δ(*lac-pro*), *sup*E, *thi, hsd*D5/F′ *tra*D36, *pro* A$^+$ B$^+$, *lac*I^{q}ZΔM15) [14]. *C. acetobutylicum* was routinely subcultured in 2 × YT broth (per litre: bactotryptone, 16 g, yeast extract, 10 g, NaCl, 4 g) supplemented with 0.5% (w/v) glucose. Leucine auxotrophy was determined on CMM [1]. Other methods have been published elsewhere [15].

3.2. DNA isolation and characterisation

Plasmid DNA was isolated from *E. coli* on a small scale (0.5 ml) by the method of Holmes and Quigley [16]. The method used for larger scale (500 ml) preparations has been previously described [15]. Plasmid DNA was isolated from *C. acetobutylicum* by the method of Trieu-Cuot et al. [17] using the cell pellet collected from 5 ml of overnight broth culture as the starting material. Also the suspension was extracted once with an equal volume of chloroform : iso-amyl alcohol (24 : 1, vol : vol) after phenol/chloroform extraction. This appeared to improve restriction enzyme digestion of the purified DNA.

3.3. Electroporation

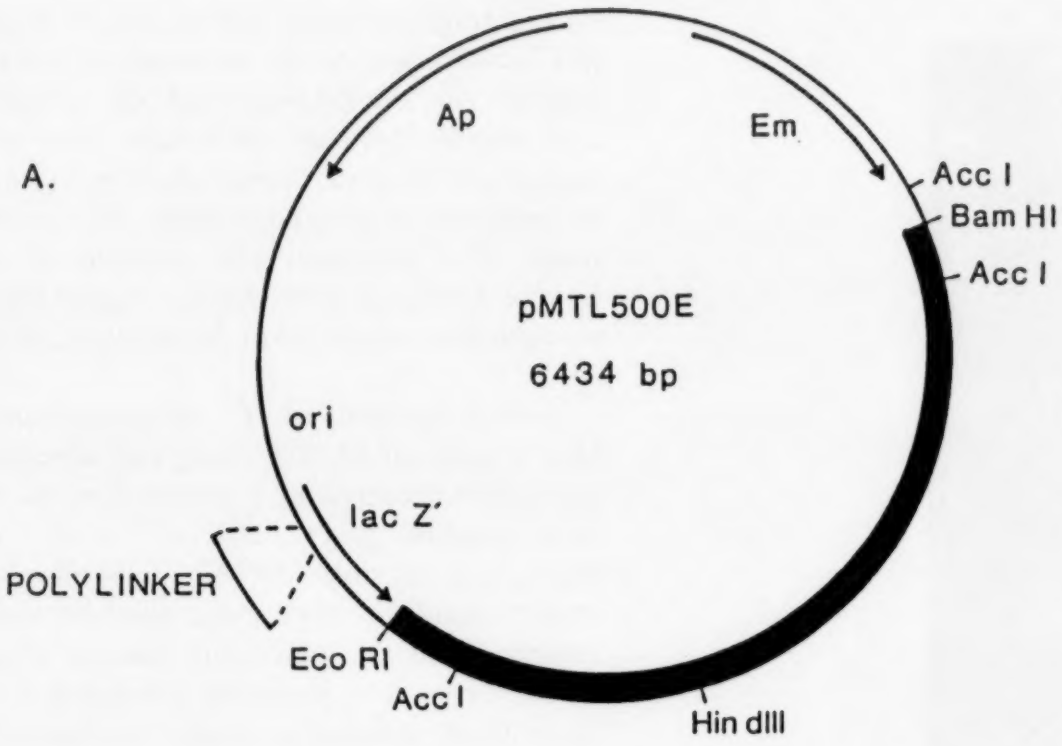
Cells for electroporation were grown in 100 ml of glucose supplemented (0.5% w/v) 2 × YT broth to mid-exponential phase ($A_{600} = 0.6$) prior to processing for electroporation according to the method of Dower [18]. This basically involved harvesting cells by centrifugation, washing once in 10 ml of cooled (4° C) electroporation buffer (270 mM sucrose, 1 mM $MgCl_2$, 7 mM $NaHPO_4$, pH 7.4) and resuspending in 5 ml cooled electroporation buffer. After holding the cell suspension on ice for 10 min approximately 0.5 μg of DNA was added to 0.8 ml of the cell suspension in Bio-Rad electroporation cuvettes and the cells cooled on ice for a further 8 min prior to electroporation in a Bio-Rad Gene Pulser™. The electric pulse applied was 0–2.5 kV (0–6.25 kV/cm in 0.4 cm cuvettes). The cells were again held on ice for 10 min before being diluted (0.1 ml cells in 0.8 ml 2 × YT + glucose) and incubated at 37° C for one hour to allow gene expression prior to plating onto selective and non-selective plates. Transformant colonies were usually visible after 16–24 h but plates were generally incubated for 2–3 days before being counted. Manipulations were carried out in an anaerobic cabinet (Anaerobic Workstation Mark III, Don Whitley Ltd, Yorkshire U.K.) with exposure to air during centrifugation (in sealed tubes) and electroporation kept to a minimum.

4. RESULTS

4.1. Construction of a cloning vector

To evaluate the potential of electroporation as a procedure for introducing genetic material into *C. acetobutylicum* a suitable plasmid vector was required. Such a plasmid had to be capable of replication and expression of selectable antibiotic resistance in the clostridial host. Since no such small genetically marked plasmid exists in *C. acetobutylicum* a plasmid, pMTL500E, was constructed by cloning the replication origin and erythromycin resistance gene [19] of the streptococcal plasmid pAMβ1 [20] into the *E. coli* cloning vector pMTL20 [21] (Fig. 1). Plasmid pAMβ1 has previously been shown both to replicate, and to express erythromycin resistance, in *C. acetobutylicum* [1,2]. The points of insertion of the pAMβ1-derived DNA into pMTL20 were chosen such that there was no impairment of plasmid encoded functions. Plasmid pMTL500E was therefore capable of replicating in *E. coli* as well as *C. acetobutylicum* (and many other Gram-positive species) and retained the cloning advantages of pMTL20, viz: cloning of foreign DNA into its extensive polylinker region causes insertional in-

A.



B.

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                                    AccI
                          SmaI      SalI                    XhoI
          EcoRI SstI  KpnI     BamHI  XbaI     AatII MluI  NcoI  BglII   StuI PstI SphI  HindIII
ATGACCATGATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACGTCACGCGTCCATGGAGATCTCGAGGCCTGCAGGCATGCAAGCTTGGCACTGGCC
 M  T  M  I  T                                                                                 L  A
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Fig. 1. Construction of a *C. acetobutylicum/E. coli* shuttle vector. (A) pMTL500E was constructed by insertion of the erythromycin resistance of plasmid pAMβ1 [19], as a blunt-ended *Hha*I fragment, into the unique *Eco*RV site in the backbone of plasmid pMTL20 [21]. The replication origin of pAMB1 (*Eco*RI/*Hpa*I fragment, [20]) (shown as a bold line) was converted to *Eco*RI/*Bam*HI by the addition of the sequence 5′-GGGGATCC-3′ to the *Hpa*I end. This fragment was blunt ended [3] and inserted into the unique *Nhe*I site in the backbone of the above recombinant plasmid. (B) The polylinker sequence of pMTL20 [21]. The encoded amino acid of *LacZ′* is indicated below the illustrated nucleotide sequence. The single letter amino acid code is used beneath the second nucleotide of the appropriate codon.

activation of the *lacZ′* gene which can be detected in suitable *E. coli* strains (e.g. TG1 and JM83, see MATERIALS AND METHODS on media supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), [21].

4.2. Plasmid transfer by electroporation

All electroporation-mediated transformation experiments were performed using the Bio-Rad Gene Pulser™ set at its maximum capacitance of 25 μFD. At this capacitance voltages between 0.5 and 6.25 kV/cm were applied to *C. acetobutylicum* cells held in 4 mm cuvettes using the procedure outlined in MATERIALS AND METHODS. Transformants were obtained at voltages above 2.5 kV/cm up to the maximum 6.25 kV/cm. The frequency of transformation was low and inconsistent, as was the level of survival of colonies at the various voltages used. The most consistent transformation occurred at 5.0 kV/cm (giving time constants between 4.7 and 6.9 ms, and cell survival between 0.7% and 23% under the conditions used). At this voltage the frequency of transfer of pMTL500E varied between 8.0×10^1 and 2.9×10^3 transfor-

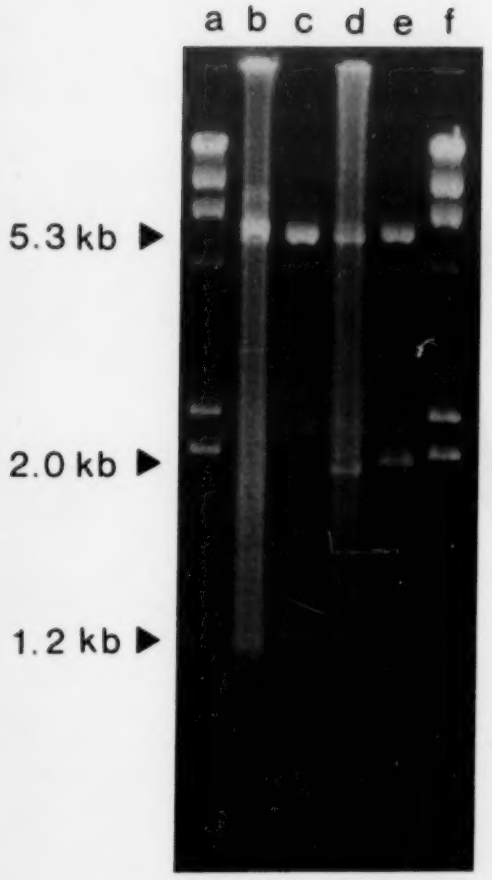

Fig. 2. Restriction analysis of *C. acetobutylicum* transformants. Lanes a and f, phage lambda, *Hin*dIII; lane b, *C. aceto.* (pMTL500E, *Hin*dIII); lane c, pMTL500E, *Hin*dIII; lane d, *C. aceto.* (pLEU100, *Hin*dIII); lane e, pLEU100, *Hin*dIII. Arrows refer to sizes of common restriction fragments. Slight apparent size differences between *E. coli* and *C. acetobutylicum* bands may be due to differences in the purity of the DNA.

mants/μg plasmid DNA. Transformants were never seen in the absence of an electrical pulse. Plasmid DNA was extracted from erythromycin resistant colonies and shown to be indistinguishable from native pMTL500E on the basis of agarose gel electrophoresis (Fig. 2, lanes b and c).

4.3. Gene transfer by electroporation

Having established that electroporation could be used to introduce plasmids into *C. acetobutylicum* the possibility that pMTL500E might be used as a vehicle for the introduction of heterologous genes was investigated. Previous work in this laboratory has shown that a 2.2 kb *Sph*I/*Cla*I chromosomal fragment derived from *C. pasteurianum* ATCC 6013 complemented a *leu*$^-$ auxotrophic mutant of *C. acetobutylicum,* SBA9, when introduced by cointegrate conjugal transfer [22]. As this fragment could not be cloned directly into pMTL500E, due to the presence of *Cla*I restriction sites in the pAMβ1 replicon moiety, it was first inserted between the unique *Sph*I and *Acc*I sites of pMTL20 [21] from which position it could be removed as a similar sized *Xba*I/*Sph*I fragment. This fragment was introduced into the unique *Xba*I and *Sph*I sites in plasmid pMTL500E to yield the recombinant plasmid pLEU100 (Fig. 3).

Transformation of *C. acetobutylicum* SBA9 (*leu*$^-$) with pLEU100 using the electroporation procedure occurred at a similar rate to that seen with plasmid pMTL500E (1.1×10^3 transformants/μg plasmid DNA). Subculture of the transformants demonstrated coinheritance of the erythromycin resistance and leucine prototrophy in each case and plasmids, extracted from overnight broth cultures of these transformants, were shown to be identical in size to the parental plasmid by agarose gel electrophoresis (Fig. 2, lanes d and e).

5. DISCUSSION

The electroporation procedure outlined above provides a rapid and reliable means for the transformation of whole cells of *C. acetobutylicum* with plasmid DNA. The low frequency of transformation observed rules out the current protocol for shotgun cloning of manipulated plasmid DNA directly into *C. acetobutylicum.* This will impose obvious limitations upon the investigation of genes which are not expressed in *E. coli* (such as sporulation genes), however, electroporation can make use of bifunctional shuttle vectors to transfer recombinant DNA which has been characterised in *E. coli* into *C. acetobutylicum.* This method of gene cloning has been used very successfully with other Gram-positive organisms (e.g. *B. subtilis, S. lactis*).

The shuttle vector pMTL500E should prove to be particularly useful as it possesses multiple cloning sites within the plasmid encoded *lacZ'* gene, facilitating recombinant detection in *E. coli.* Furthermore the broad host-range of plasmid pAMβ1 implies that pMTL500E may be applicable as a

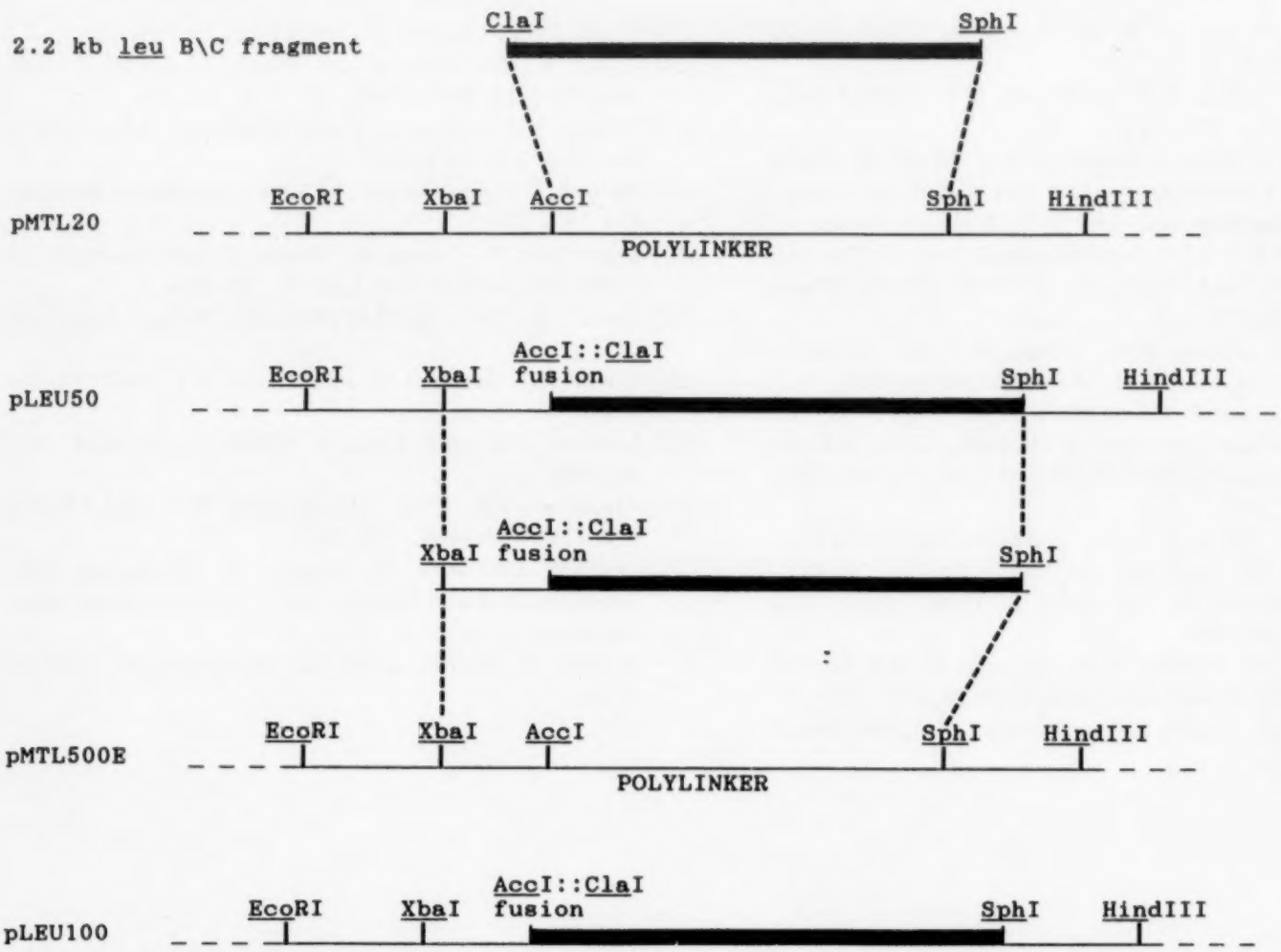

Fig. 3. Construction of the recombinant plasmid pLEU100. The 2.2 *Cla*I/*Sph*I fragment from the *C. pasteurianum* chromosome which carries genes complementing *leuB* and *leuC* mutations in *E. coli* [22] was inserted into the complementary *Acc*I and *Sph*I sites of pMTL20 [21]. The fragment was then relased as an *Xba*I/*Sph*I fragment which was inserted into the complementary sites in plasmid pMTL500E.

cloning vector to many Gram-positive genera. It has recently been postulated that the observed structural instability of recombinant plasmids cloned in *Bacillus subtilis* may be attributable to the method of replication adopted by the commonly used cloning vectors. This replication strategy involves the generation of single stranded DNA intermediates as part of a rolling circle mechanism [23]. DNA fragments cloned in plasmids with pAMβ1-based replicons, which replicate without the generation of such intermediates have proved to be dramatically more stable in *B. subtilis* (S.D. Ehrlich, personal communication) and hence vectors based upon the pAMβ1 replicon may become those of choice for most Gram-positive work.

It may be further noted that the electroporation procedure should be generally applicable to plasmid vectors capable of replication in *C. acetobutylicum* and indeed is being currently used to determine the ability of commonly used Gram-positive vectors to replicate in that host.

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REFERENCES

[1] Oultram, J.D. and Young, M. (1985) FEMS Microbiol. Lett. 27, 129–134.

[2] Oultram, J.D., Davies, A. and Young, M. (1987) FEMS Microbiol. Lett. 42, 113–119.
[3] Jones, D.T., Jones, W.A. and Woods, D.R. (1985) J. Gen. Microbiol. 131, 1213–1216.
[4] Reysset, G., Hubert, J., Podvin, L. and Sebald, M. (1988) Anaerobes Today. (Hardie, J.M. and Boriello, S.P., eds.), pp. 239, John Wiley and Sons Ltd, Chichester, Sussex.
[5] MacNeil, D.J. (1987) FEMS Microbiol. Lett. 42, 239–244.
[6] Chassy, B.M. and Flickinger, J.L. (1987) FEMS Microbiol. Lett. 44, 173–177.
[7] Powell, I.B., Achen, M.G., Hillier, A.J. and Davidson, B.E. (1988) Appl. Environ. Microbiol. 54, 655–660.
[8] Somkuti, G. and Steinberg, D.H. (1987) Eur. Proc. Eur. Cong. on Biotechnol. Vol. 1 (Neijssel, O.M., van der Meer, R.R. and Luyben, K.Ch.A.M., eds.), p. 412, Elsevier, New York.
[9] Miller, J.F., Dower, W.J. and Tompkins, L.S. (1988) Proc. Natl. Acad. Sci. USA. 85, 856–860.
[10] Ito, K., Nishida, T. and Izaki, K. (1988) Agric. Biol. Chem. 52, 293–294.
[11] Hashimoto, H., Morikawa, H., Yamada, Y. and Kimura, A. (1985) Appl. Microbiol. Biotechnol. 21, 336–339.
[12] Fromm, M.E., Taylor, L.P. and Walbot, V. (1986) Nature 319, 791–793.
[13] Vieira, J. and Messing, J. (1982) Gene 19, 259–268.
[14] Carter, P., Bedouelle, H. and Winter, G. (1985) Nucleic Acids Res. 13, 4431–4443.
[15] Minton, N.P., Atkinson, T. and Sherwood, R.F. (1983) J. Bacteriol. 156, 1222–1227.
[16] Holmes, D.S. and Quigley, M. (1981) Analytical Biochem. 114, 193–197.
[17] Trieu-Cuot, P., Carlier, C., Martin, P. and Courvalin, P. (1987) FEMS Microbiol. Lett. 48, 289–294.
[18] Dower, B. (1987) Bio-Rad Molecular Biology Reports 1, 5.
[19] Brehm, J.K., Salmond, G. and Minton, N.P. (1987) Nucleic Acids Res. 15, 3177.
[20] LeBlanc, D.J. and Lee,L.N. (1984) J. Bacteriol. 157, 445–453.
[21] Chambers, S.P., Prior, S.E., Barstow, D.A. and Minton, N.P. (1988) Gene 68, 139–149.
[22] Oultram, J.D., Peck, H., Brehm, J.K., Thompson, D.E., Swinfield, T.-J. and Minton, N.P. (1988) Mol. Gen. Genet. (In press).
[23] te Riele, H., Michel, B. and Ehrlich, S.D. (1986) EMBO J. 5, 631–637.

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On the presence of calmodulin-like protein in mycobacteria

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1. SUMMARY

A heat-stable factor, antigenically cross-reacting with calmodulin, was found mainly in the soluble fraction of *Mycobacterium smegmatis* ATTC 14468 and *Mycobacterium bovis* BCG 14 004 001. Maximum amounts of calmodulin were found in the early logarithmic phase of growth. The cell-free extract of *M. smegmatis* did not stimulate, rather inhibited cAMP phosphodiesterase due to the presence of certain endogenous inhibitory protein(s). However, the cAMP phosphodiesterase stimulation could be observed, after the removal of inhibitor(s). This is the first report indicating the presence of calmodulin in mycobacteria, a member of the prokaryotes.

2. INTRODUCTION

Calmodulin has now been established as the primary receptor for calcium in eukaryotic cells [1]. It is a small, heat- and acid-stable protein whose aminoacid sequence has been conserved almost perfectly throughout evolution [2]. Calmodulin–calcium complex regulates a variety of enzymes including nucleotide phosphodiesterase [3], adenylate cyclase [4], ATPase [5] and calcium pump of erythrocyte plasma membrane [6]. Calmodulin has been found in many eukaryotic cells [1,2] including *Saccharomyces cerevisiae* [7]. Iwasa et al. [8] suggested that calmodulin-like activity existed in *Escherichia coli* although Chafoules et al. [9] did not find any protein immunologically similar to calmodulin in *E. coli.* Calmodulin like protein has recently been reported in *Bacillus subtilis* [10], *Streptomyces erythraeus* [11] and *Myxococcus xanthus* [12]. Calmodulin activity was identified in purified feline leukemia virus [13]. High-affinity Ca^{2+} binding proteins of 33–60 kDa from *E. coli* did not stimulate calmodulin regulated enzymes [14]. According to Grand et al. [15] and Clarke et al. [16] prokaryotes appear to lack calmodulin. Although Wolf et al. [17] found a calmodulin activatable adenylate cyclase in membranes from the prokaryote *Bordetella pertussis.*

In the present communication, presence of calmodulin has been demonstrated in mycobacteria by using sensitive and specific radioimmunoassay. The data indicate the presence of

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inhibitor(s) in the crude cell-free extract inhibiting commonly used cAMP phosphodiesterase assay of calmodulin. Calmodulin activity in terms of cAMP phosphodiesterase stimulation could be demonstrated after removing the inhibitory proteins.

3. MATERIALS AND METHODS

Growth of mycobacteria: *M. smegmatis* ATCC 14468 and *M. bovis* 14 004 001 (from Pasteur Institute) were grown on Youmans and Karlson medium [18] as static culture at 37 °C, as described earlier [19]. Cells were harvested at desired growth phase according to the experimental protocol.

Harvested cells were washed twice with distilled water and resuspended in 50 mM Tris-Cl buffer, pH 7.8 containing 3 mM $MgSO_4$, 1 mM DTT and 1 mM EGTA. These cells were sonicated at 30–40 KHz for 3 min. This operation was repeated three times. The homogenate was centrifuged at 20 000 $\times g$ for 20 min. The supernatant so obtained was subjected to ultracentrifugation at 105 000 $\times g$ for 90 min. The pellet obtained was resuspended in the above mentioned buffer. All these fractions were kept in a boiling water bath for 4 min chilled immediately in ice-cold acetone, and then centrifuged to remove denatured proteins.

Calmodulin was assayed by its capacity to stimulate calmodulin deficient cyclic AMP phosphodiesterase [20]. The reaction mixture contained 4 mM Tris-Cl, pH 8, 50 μM $CaCl_2$, 5 mM $MgSO_4$ and 60 μg calmodulin deficient cAMP phosphodiesterase and an appropriate amount of extract in a total volume of 0.1 ml. The reaction was started by addition of ^{3}H-cAMP (2 mM, 0.1 μCi). After 10 min incubation at 30 °C, the reaction was terminated by placing the tubes in a boiling water bath for 2 min. Samples were incubated with 5′-nucleotidase (50 μg) at 30 °C for 10 min. ^{3}H-Adenosine was separated from unreacted substrate using Dowex-1 $\times$ 8 anion exchange resin [21]. Calmodulin was estimated by ^{125}I radioimmunoassay kit supplied by NEN [22]. An increasing amount of bovine brain calmodulin as well as mycobacterial extract when assayed by radioimmunoassay showed similar slopes. Purified bovine brain calmodulin was a generous gift of Prof. D. Marme, Biology Institute III, Freiburg University, F.R.G. Affinity purified antibodies were kindly provided by Prof. A.R. Means.

All other chemical were obtained from Sigma Chem. Co., St. Louis, MO. Cyclic-(8-^{3}H)-adenosine 3′-5′-monophosphate was obtained from Radiochemical Center, Amersham.

4. RESULTS

The levels of calmodulin in *M. smegmatis* and *M. bovis* BCG as determined by radioimmunoassay are shown in Table 1. *M. smegmatis* contains a higher amount of calmodulin than *M. bovis* BCG throughout the growth period, though the highest concentration was present in the early logarithmic phase of growth in both cases. There is a sharp decrease in the levels of calmodulin at the stationary phase.

The subcellular distribution of calmodulin in *M. smegmatis* is shown in Table 2. About 17% of the total calmodulin was found to be associated with the particulate fraction (105 000 $\times g$ pellet). The rest of the calmodulin was present in the soluble fraction.

Heat-inactivated crude cell-free extract of *M. smegmatis* was also assayed for calmodulin by commonly used cAMP phosphodiesterase stimulation. Surprisingly, cAMP phosphodiesterase was not stimulated by mycobacterial calmodulin, rather, 66% inhibition was noticed in the basal activity of cAMP phosphodiesterase (Table 3). Even the addition of purified bovine brain

Table 1

Levels of calmodulin in *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG during various phases of growth

Growth phase	*M. smegmatis*	*M. bovis* BCG
	(Calmodulin ng/g dry weight)	
Early log phase	257.35	153.22
Mid log phase	202.22	83.30
Late log phase	200.80	85.86
Stationary phase	95.80	65.20

Cells were grown to the desired phase in Youmans and Karlson medium. Cell free extract was prepared as described in MATERIALS AND METHODS. Calmodulin was estimated by radioimmunoassay.

Table 2

Localization of calmodulin in various cell fractions of *Mycobacterium smegmatis* ATCC 14469

Fractions	Calmodulin (% of cell homogenate)
Cell homogenate	100 *
20000 × *g* pellet	ND
20000 × *g* supernatant	99.8
105000 × *g* pellet	17.1
105000 × *g* supernatant	82.2

* 202.2 ng/g dry weight
ND = Not detectable
Cells were grown to mid log phase in Youmans and Karlson medium. Fractionation of crude extract is given under MATERIALS AND METHODS. Calmodulin was estimated by radioimmunoassay.

calmodulin to this assay system could not alter the inhibition which prompted us to look for a possible presence of inhibitor(s) in the cell free extract of *M. smegmatis*. For this, the extract was subjected to ammonium sulfate fractionation. Three fractions were obtained, namely fraction I, II and III (up to 35%, 65% and 100% saturation respectively). After dialysis, these fractions were added to bovine cAMP phosphodiesterase and assayed for its activity. Fraction I and II were found to inhibit cAMP phosphodiesterase activity to the extent of 98% and 97% respectively while fraction III was found to stimulate it by 100% (Table 3). Similar results were obtained by using 105000 × *g* supernatant instead of crude extract (data not shown). Further, when purified bovine brain calmodulin was added to these fractions along with phosphodiesterase, fraction III stimulated phosphodiesterase activity by 280%. Trifluoperazine, W7 (known inhibitors of calmodulin) as well as affinity-purified antibodies when incubated with fraction III abolished this stimulation.

Table 3

Effect of ammonium sulphate fractionated proteins on cAMP phosphodiesterase

Additions	Enzyme activity (ng cAMP hydrolysed/min)	
	− Calmodulin	+ Calmodulin
None [a]	75.00	275.50 *
Crude extract	17.30	11.00 **
Fraction I	1.13	4.85 **
Fraction II	2.40	4.35 **
Fraction III	152.50	423.30 *
Fraction III + TFP	80.00	82.70
Fraction III + W7	78.00	80.50
Fraction III + CaM Ab	72.00	70.00

[a] Only cAMP phosphodiesterase, TFP-trifluoperazine.
* $p > 0.01$, ** $p > 0.05$.
Cell free extract was prepared as mentioned in MATERIALS AND METHODS. It was subjected to ammonium sulphate fractionation. Fraction I (0–35%), fraction II (35–65%) and fraction III (65–100%) saturation. Precipitates obtained were dissolved in assay buffer, dialysed, kept at boiling water bath for 4 min and chilled immediately. Fifty microgram protein from each fraction was added to tubes and assayed for cAMP phosphodiesterase. Calmodulin (800 ng), TFP (50 μM), W7 (50 μM) and calmodulin antibodies (100 μg) were added where indicated.

5. DISCUSSION

Calmodulin regulates a variety of physiological processes, which suggests that the intracellular level of this Ca^{2+} receptor could be an important mechanism in controlling cell growth and metabolism [2]. Highest levels of calmodulin were in the early logarithmic phase of growth in *M. smegmatis* as well as in *M. bovis* BCG, during the time when cells were actively dividing, suggesting its possible role in the induction of cell proliferation. Calmodulin requirement in the early phase of growth seems to be more due to relatively greater metabolic activity. Under these conditions the amount of calmodulin found in *M. smegmatis* is more than that of *M. bovis* BCG, most probably due to the relatively fast growing nature of *M. smegmatis*. Transformed animal cells are generally found to have an increased level of cellular calmodulin [23]. Calmodulin has also been proposed to be involved in DNA repair perhaps at the time of initiation of DNA synthesis [24].

In *M. smegmatis* calmodulin was found mainly in the soluble fraction (82%) whereas 17% of the total was associated with membrane fraction. In rat brain 20% of calmodulin is membrane bound [25]. In C-6 glial tumor cells the particulate calmodulin (26% total) showed 6%, 11% and 8% association with nuclear, mitochondrial and microsomal fraction respectively [26]. Distribution of

calmodulin does not correspond to that of Ca^{2+} activatable phosphodiesterase [27] which suggests that calmodulin serves many regulatory roles and that regulation of cAMP phosphodiesterase is only one of them. In the case of *M. smegmatis* guanylate cyclase [28] and cAMP phosphodiesterase [29] have been reported to be present in soluble forms whereas ATPase [30] adenylate cyclase [31] and cGMP phosphodiesterase [32] are located in particulate fraction.

Inhibition of cAMP phosphodiesterase activity by crude cell-free extract indicates towards the possible presence of a factor which inhibits the assay probably by inhibiting cAMP phosphodiesterase. Alternatively, this factor, by binding to cAMP phosphodiesterase, changes its conformation, which in turn cannot bind to calmodulin any more. Ammonium sulphate fractionation followed by dialysis revealed that the inhibitory factor is nondialysable and possibly a protein. Exogenously added calmodulin could not overcome the inhibition caused by crude cell-free extract. Out of three fractions (I, II and III) isolated by ammonium sulphate fraction, only fraction III did not possess inhibitory property rather it stimulated cAMP phosphodiesterase two fold. These data indicate that the inhibitor is precipitated in fraction I and II whereas calmodulin is concentrated in fraction III. Addition of calmodulin to fraction III could further enhance the cAMP phosphodiesterase activity. Trifluoperazine as well as W7, when added to the assay system along with the fraction III, abolished the stimulation. Affinity-purified antibodies against calmodulin also had the same effect. All these data are in favor of having at least one protein immunologically as well as functionally similar to calmodulin in fraction III. A heat labile inhibitor protein of cAMP metabolizing enzymes has been identified in bovine brain [33]. A natural inhibitor of calmodulin has recently been reported [34] besides many synthetic antipsychotic drugs, which act by inhibiting calmodulin activity and thereby inhibit cAMP phosphodiesterase.

The results presented in this study indicate towards the presence of calmodulin like protein in mycobacteria for the first time. Radioimmunoassay has been used for the estimation of calmodulin which is not only more sensitive than Ca^{2+} dependent cAMP phosphodiesterase activation assay but also affords the detection of calmodulin on the basis of antigenic determinants and thus measures calmodulin in terms of its structure. Further, the accuracy of radioimmunoassay is not affected by the presence of heat-stable inhibitory protein(s), which affects the utility of enzyme assay. It is tempting to presume that because of such inhibitor(s), calmodulin was not reported in prokaryotes [6,7]. It is also suggested that during evolution nature provided calmodulin to mycobacteria, which lie near the borderline of eukaryotes and prokaryotes. Further attempts are being made to isolate and characterize calmodulin and the inhibitory protein(s) from *M. smegmatis*. The inhibitory protein(s) might be involved in the regulation of cAMP dependent cell functions by modulating cAMP phosphodiesterase in mycobacteria.

REFERENCES

[1] Means, A.R., Tash, J.S. and Chafouleas, J.G. (1982) Physiol. Rev. 62, 1–39.

[2] Klee, C.B., Crouch, T.H. and Richman, P.G. (1980) Ann. Rev. Biochem. 49, 489–515.

[3] Cheung, W.Y. (1970) Biochem. Biophys. Res. Commun. 38, 533–538.

[4] Brostrom, C.O., Brostrom, M.A. and Wolff, D.J. (1977) J. Biol. Chem. 252, 5677–5685.

[5] Gopinath, R.M. and Vincenzi, F.F. (1977) Biochem. Biophys. Res. Commun. 77, 1203–1209.

[6] Jarrett, H.W., Penniston, J.T. (1977) Biochem. Biophys. Res. Commun. 77, 1210–1216.

[7] Hubbard, M., Brandley, M., Sullivan, P., Shepherd, M. and Forrester, I. (1982) FEBS Lett. 137, 85–88.

[8] Iwasa, Y., Yonemitsu, K., Matsui, K., Fukunaga, K. and Miyamoto, E. (1981) Biochem. Biophys. Res. Commun. 98, 656–660.

[9] Chafouleas, J.G., Dedman, J.R., Munjaal, R.P. and Means, A.R. (1979) J. Biol. Chem. 254, 10262–10267.

[10] Fry, I.J., Villa, L., Kuehn, G.D. and Hageman, J.H. (1986) Biochem. Biophys. Res. Commun. 134, 214–217.

[11] Swan, D.G., Hale, R.S., Dhillon, N. and Leadlay, P.F. (1987) Nature 329, 84–85.

[12] Inouye, S., Franceshini, T. and Inouye, M. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 6829–6833.

[13] Lewis, M.G., Chang, J.Y., Olsen, R.G. and Fertel, R.H. (1986) Biochem. Biophys. Res. Commun. 141, 1077–1083.

[14] Harmon, A.C., Prasher, D. and Cromier, M.J. (1985) Biochem. Biophys. Res. Commun. 127, 31–36.

[15] Grand, R.J.A., Narain, A.C. and Perry, V.S. (1980) Biochem. J. 185, 755–760.

[16] Clarke, M., Bazari, W.L. and Kayman, S.C. (1980) J. Bact. 141, 397–400.
[17] Wolf, J., Hopecook, G., Goldhammer, A.R. and Berkowitz, S.A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3841–3844.
[18] Youmans, G.P. and Karlson, A.G. (1947) Am. Rev. Tuberc. 55, 529–535.
[19] Dhariwal, K.R., Chander, A. and Venkitasubramanian, T.A. (1978) Arch. Microbiol. 116, 69–75.
[20] Cheung, W.Y. (1971) J. Biol. Chem. 246, 2859–2869.
[21] Schultz, G., Bohme, E. and Hardman, J.G. (1978) Methods Enzymol. 38, 9–13.
[22] Dedman, J.R., Welsh, M.J. and Means, A.R. (1978) J. Biol. Chem. 253, 7515–7521.
[23] Watterson, D.M., Eldik, L.J.V., Smith, R.E. and Vanaman, T.C. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2711–2715.
[24] Chafouleas, J.G., Balton, W.E. and Means, A.R. (1984) Science 224, 1346–1348.
[25] Teshima, Y. and Kakiuchi, S. (1978) J. Cyclic Nucleotides Res. 4, 219–231.
[26] Brostrom, C.O. and Wolff, D.J. (1974) Arch. Biochem. Biophys. 165, 715–727.
[27] Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D. and Means, A.R. (1977) J. Biol. Chem. 252, 8415–8422.
[28] Bhatnagar, N.B., Bhatnagar, R. and Venkitasubramanian, T.A. (1984) Biochem. Biophys. Res. Commun. 121, 634–640.
[29] Lee, C.H. (1978) J. Gen. Microbiol. 107, 177–181.
[30] Grover, G., Dhariwal, K.R. and Venkitasubramanian, T.A. (1978) J. Gen. Microbiol. 105, 343–345.
[31] Padh, H. and Venkitasubramanian, T.A. (1979) Ind. J. Biochem. Biophys. 16, 131–135.
[32] Lee, C.H. (1978) J. Gen. Microbiol. 108, 325–328.
[33] Wallace, R.W., Lynch, T.J., Tallant, E.A. and Cheung, W.Y. (1979) J. Biol. Chem. 254, 377–382.
[34] Leung, P.C., Taylor, W.A., Wang, J.H. and Tipson, C.L. (1984) J. Biol. Chem. 259, 2742–2747.

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Transformation of group A streptococci by electroporation

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1. SUMMARY

The introduction, via electroporation, of free plasmid DNA into three strains of *Streptococcus pyogenes* is described. The method is very simple and rapid and efficiencies vary from 1×10^3 to 4×10^4 per μg of DNA. The method was also used to introduce an integrative plasmid and transformants were obtained, albeit at a somewhat lower frequency (2×10^2). Some of the plasmids used in this study are derivatives of the *Lactococcus lactis* subsp. *cremoris* Wg2 plasmid pWV01. These broad host range vectors replicate in Gram-positives as well as Gram-negatives (viz. *Escherichia coli*). Here we show that they also replicate in *S. pyogenes* and *S. sanguis.*

2. INTRODUCTION

Group A streptococci (*Streptococcus pyogenes*) are important human pathogens causing a variety of serious inflammatory diseases, sometimes followed by acute rheumatic fever or poststreptococcal glomerulonephritis. Their importance in view of medical care has resulted in an intensification of research aimed at the elucidation of the genetic background of these organisms. Only recently, this has led to the identification, cloning and sequencing of a number of genes, amongst which several M protein genes [1–5], the genes for streptolysin [6] and streptococcal exotoxin A [7,8], the Immunoglobulin G-receptor gene [9], and the hyaluronidase gene from an *S. pyogenes* bacteriophage [10]. A major drawback in the genetic research in this field is the fact that no direct system for transformation of group A streptococci is available. Transduction and conjugation have been shown to operate in these organisms (for a review see Wannamaker [11], and [12]). Using conjugative mobilization, Scott et al. [13] were able to transfer the *emm*6 gene, coding for type 6 M protein, to an M^- strain of *S. pyogenes,* which became functionally M^+.

The development of a transformation system for group A streptococci is essential, however, for further rapid increase in the knowledge of the factors involved in virulence and pathogenicity. This would offer the possibility of applying recombinant DNA technologies to the group A streptococci, without using an intermediate host, and would allow studies of protein and gene structure and function in the natural environment. In this communication we demonstrate the uptake of free plasmid DNA by two strains of *S. pyogenes* using the electroporation technique, a method suc-

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cessfully applied to an increasing number of microorganisms [14–20]. In addition, we show that vectors based on the cryptic *Lactococcus lactis* subsp. *cremoris* plasmid pWV01 are able to replicate in group A and H (*S. sanguis*) streptococci. Because these vectors also replicate in *Escherichia coli* and *Bacillus subtilis* [21], these observations open a new avenue for future genetic studies in group A streptococci.

3. MATERIALS AND METHODS

3.1. Strains, plasmids and media

The strains and plasmids used in this study are summarized in Table 1. All strains were routinely grown at 37°C in Brain Heart Infusion (BHI) broth with 5% horse serum (both from Difco, U.S.A.). Plasmids were isolated on a miniprep scale essentially as described by Birnboim and Doly [22]. To digest the cell wall of overnight cultures, the cells were incubated for 20 min at 37°C in lysis solution containing 10 mg/ml lysozyme and 30 U/ml mutanolysin. Agarose gel electrophoresis and restriction enzyme digestions were done as described by Maniatis et al. [23].

Table 1

Strains and plasmids used in this work

Strain	Phenotype	Source
Streptococcus pyogenes (Lancefield group A)		
154	Tet^R, Rif^R	Lab collection, Leningrad
1800	M18	Lab collection, Leningrad
CS112	M^-, Str^R	P. Cleary, University of Minnesota, USA
Streptococcus sanguis (Lancefield group H)		
challis 57		H. Malke, CIMET, Jena, GDR
challis GS10.1		Lab collection, Leningrad
Plasmid (size in kb)	**Marker [a]**	**Reference**
pGK12 (4.5)	Em^R, Cm^R	[21]
pGK13 (4.9)	Em^R, Cm^R	pGK12 derivative [21] Lab collection, Groningen
pGK20 (5.9)	Em^R, Cm^R	pGK13 with *lacZ* gene fusion, Lab collection, Groningen
pVI101 (7.4)	Em^R	Golubkov et al. [28]
pGT128 (5.4)	Em^R	Golubkov et al. [24]
pPPCV5 (4.3)	Em^R	Lab collection, Leningrad

[a] Em^R: erythromycin resistance; Cm^R: chloramphenicol resistance.

3.2. Transformation

Transformation of *Streptococcus sanguis* was done according to Golubkov et al. [24] except that *S. sanguis* was grown in BHI without sheep erythrocytes.

3.3. Electroporation

Electroporation was done essentially as described by van der Lelie et al. [19]. An overnight culture of *Streptococcus pyogenes* in Todd Hewitt Broth (Difco, U.S.A.) supplemented with 40 mM dl-threonin was diluted 100-fold in the same medium and grown to an optical density at 650 nm of 0.2 ($5–8 \times 10^8$ cells per ml). For electroporation, the cells in 25 ml of the culture were washed twice in electroporation buffer (EB: 5 mM phosphate buffer, pH 6.9, 0.3 M sucrose, 1 mM $MgCl_2$), resuspended in 2 ml EB and cooled on ice for at least 15 min. DNA was added to 0.6 ml of the cell suspension and electroporation was carried out at 2500 V, 25 μF in a Gene Pulser™ (Bio-Rad Laboratories). After the electric pulse, the cells were kept on ice for 10 min and subsequently diluted 10-fold in BHI, containing 0.3 M sucrose and 5% horse serum. After allowing time for expression (2 h at 37°C), the cells were plated onto BHI-agar plates containing 5 μg of erythromycin per ml. Transformant colonies were visible after 48 h of incubation at 37°C.

4. RESULTS

4.1. Electroporation

To investigate whether electroporation was possible in group A streptococci, *Streptococcus pyogenes* strain 154 was chosen as the model strain. Various plasmids were used as donor molecules. These are listed in Table 1 and all carry an erythromycin resistance gene as the selectable marker. After the first pilot experiments, in which we found some Em^R colonies with plasmid pGK13, we decided to use this plasmid, a broad host range

vector derived from a cryptic plasmid of *Lactococcus lactis* subsp. *cremoris* [21], to optimize the system parameters. We found that the best electroporation results for strain 154 were obtained when logarithmically growing cells were used (OD at 650 nm approximately 0.2; $5–8 \times 10^8$ cells per ml). A 100-fold diluted overnight culture of the strain usually reached this optical density after 2.5 to 3 h of growth at 37°C. The rate of survival of the cells after the high-voltage electric pulse was 40–55% in the various experiments. The number of transformants varied from 10^4 to 10^5 per μg of DNA (Table 2). With the streptococcal vectors pVI101 and pGT128 transformation frequencies of 10^3 and 5×10^3 were obtained, respectively (Table 2). Using the same conditions of electroporation, we were able also to introduce plasmid DNA in the *S. pyogenes* strains CS112 and 1800 (Table 2). Even after several trials, no transformants of strain 1800 with plasmid pGK13 were found. In all cases, the plasmid content of several transformants was checked using a plasmid minipreparation procedure. The molecular mass of the plasmids in the transformants was the same as the original plasmids used for the electroporation, as deduced from restriction enzyme analysis and subsequent agarose gel electrophoresis (Fig. 1). Furthermore, restriction enzyme analysis confirmed that no major rearrangements had occurred during or after the uptake process (results not shown). Plasmid pGK13 contains, apart from the erythromycin resistance gene, a gene conferring resistance to chloramphenicol in all the hosts it has been transferred to. Both resistance genes originate from *Staphylococcus aureus*. To investigate whether the Cm^R gene also functions in *S. pyogenes*, a culture of *S. pyogenes* strain 54(pGK13) was diluted and plated onto BHI-agar plates with increasing amounts of chloramphenicol. The Cm^R gene was expressed in this strain with a MIC value of approximately 10 μg/ml.

Fig. 1. Agarose (0.8%) gel electrophoresis of *Eco*RI digests of pVI101, (lane 1); pGT128, (lane 2); pGK13, (lane 3). Lane 4, size reference: phage lambda DNA digested with *Hin*dIII.

Table 2

Efficiencies of transformation via electroporation of group A streptococci

Plasmid (size in kb)	Strain of *S. pyogenes*		
	154	1800	CS112
pGK13 (4.9)	4×10^4	0	2×10^3
pVI101 (7.4)	5×10^3	6.5×10^3	NT
pGT128 (5.4)	1×10^3	3×10^3	NT
pPCVI1 (4.3)	NT	NT	2×10^2

NT: not tested; efficiencies are expressed as number of transformants per μg of DNA.

The integrative plasmid pPPCV5 was used in the electroporation of *S. pyogenes* strain CS112. Erythromycin resistant colonies were found with a frequency of 2×10^2 per μg DNA. No plasmid DNA was found after miniprep DNA analysis, suggesting that the plasmid had integrated into the chromosome of the recipient cells.

4.2. Transformation of S. sanguis *with* Lactococcus *derived vectors*

The cryptic plasmid pWV01 from *Lactococcus lactis* subsp. *cremoris* Wg2 has been used to construct a series of small versatile antibiotic resistance vectors [21]. The plasmids are extremely useful as they bridge the gap between Gram-posi-

tives and Gram-negatives and replicate in members of both groups (e.g. *Escherichia coli, Bacillus subtilis* and lactic acid bacteria). As shown above, derivatives of pWV01 also replicate in group A streptococci. To see whether it was possible to introduce these plasmids in the naturally transformable *Streptococcus sanguis,* we chose the challis strains 57 and GS10.1 as the recipients in transformation experiments. pWV01 derivatives pGK12, pGK13 and pGK20 were used as well as a control plasmid: pVI101. All plasmids efficiently transformed both strains to erythromycin resistance. The number of transformants per μg of DNA ranged from 1 to 2.6×10^4 for the pWV01 derivatives and was 3×10^5 for pVI101. Plasmid pGK20 is a pWV01 derivative carrying an in-frame fusion between the 9th codon of the *L. lactis* subsp. *cremoris* Wg2 proteinase gene [25] and the *E. coli lacZ* gene. Transcription and translation of the fused genes is directed by signals derived from the proteinase gene (J. Kok, unpublished). When plated on BHI-agar plates supplemented with 0.004% 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal), all the Em^R resistant transformants from pGK20 show up as blue colonies, indicative or *lacZ* gene expression and β-galactosidase activity in the *S. sanguis* transformants.

5. DISCUSSION

The lack of a method to introduce free DNA into group A streptococci is a major drawback in the dissection of the genetics of the diseases these pathogens are involved in. Until now, only one report deals with an indirect method of DNA transfer [13]. A cointegrate shuttle vector, a derivative of the *E. coli*/streptococcus plasmid pVA838, was used to transform *S. sanguis* challis. After introduction of pVA797, a conjugative plasmid which has homology with pVA838, this challis host was used as a donor in matings with an *S. pyogenes* recipient. It is clear that an efficient direct method of transformation of group A streptococci would be of eminent importance. The electroporation protocol described here is the first example of such a procedure and it offers the possibility to apply recombinant DNA technology to these organisms. Although the efficiency of transformation is only moderate, an increase can be expected soon from the optimization of the physical parameters of the electroporation equipment.

The fact that pWV01 derived vectors replicate in *S. pyogenes* offers a number of possibilities which might accelerate genetic studies in these organisms. A large number of small cloning vectors have been constructed from pWV01, some of which contain multiple cloning sites, some allowing cloning with marker inactivation (of antibiotic resistance genes). With some derivatives LacZ α-complementation is possible in *E. coli.* Some of the vectors can be used to screen for promoter and terminator sequences and a number of expression vectors have been developed (van de Guchte, pers. comm. [21,26,27]). The most important property of the vectors, however, is the fact that they replicate in Gram-positives (e.g. *Bacillus subtilis*) as well as in *E. coli.* For these two bacterial species, advanced genetic engineering techniques have been developed and, through the pWV01 vector system, these are directly linked to the nascent field of genetics of group A streptococci.

The introduction of the integrative plasmid pPPCV5 is the first step in a better understanding of the genetic organization of the chromosome of *S. pyogenes,* because it offers the possibility to isolate mutants, and, subsequently, to clone the gene(s) involved in the mutation. Furthermore, the strategy could be used to stably insert (in vitro manipulated) genes in the chromosome. In this way, the functioning of factors involved in the starting and development of pathogenicity, i.e. the role of nucleases, proteases, hyaluronidase, neuramidase, streptolysin and streptokinase can be studied in the natural environment. Ultimately, the development of efficient recombinant DNA techniques for group A streptococci could lead to the construction of artificial vaccine strains, carrying a set of antigens raising an immune response to most types of streptococci.

Only a few possibilities are mentioned here, and we are confident that the method to introduce and express free DNA in *S. pyogenes* which we describe here will be of great importance to future fundamental and applied research.

REFERENCES

[1] Scott, J.R. and Fischett, V.A. (1983) Science 221, 758–760.
[2] Hollingshead, S.K., Fischetti, V.A. and Scott, J.R. (1986) J. Biol. Chem. 261, 1677–1686,.
[3] Spanier, J. and Cleary, P. (1985) Science 225, 935–938.
[4] Kehoe, M.A., Poirier, T.P., Beachey, E.H. and Timmis, K.N. (1985) Infect. Immun. 48, 198–203.
[5] Haanes-Fritz, E., Robbins, J.C. and Cleary, P. (1987) in Streptococcal Genetics (Ferretti, J.J. and Curtiss III, R., eds.), pp. 106–108, Am. Soc. Microbiol., Washington, D.C.
[6] Kehoe, M.A. and Timmis, K.N. (1984) Infect. Immun. 43, 804–810.
[7] Weeks, C.R. and Ferretti, J.J. (1986) Infect. Immun. 52, 144–150.
[8] Johnson, L.P., L'Italien, J.J. and Schlievert, P.M. (1986) Mol. Gen. Genet. 203, 354–356.
[9] Heath, D.G. and Cleary, P.P. (1987) in Streptococcal Genetics (Ferretti, J.J. and Curtiss III, R., eds.), pp. 121–123, Am. Soc. Microbiol., Washington, D.C.
[10] Hynes, W.L. and Ferretti, J.J. (1987) in Streptococcal Genetics (Ferretti, J.J. and Curtiss III, R., eds.), pp. 150–152, Am. Soc. Microbiol., Washington, D.C.
[11] Wannamaker, L.W. (1982) in Microbiology (Schlessinger, D., ed.), pp. 112–116, Am. Soc. Microbiol., Washington, D.C.
[12] Scott, J.R., Guenthner, P.C., Malone, L.M. and Fischetti, V.A. (1986) J. Exp. Med. 164, 1641–1651.
[13] Scott, J.R., Hollingshead, S.K., Jones, K.F. and Fischetti, V.A. (1987) in Streptococcal Genetics (Ferretti, J.J. and Curtiss III, R., eds.), pp. 93–97, Am. Soc. Microbiol., Washington, D.C.
[14] Shivarova, N., Förster, W., Jacob, H.-E. and Grigorova, R. (1983) Z. Allg. Mikrobiol. 23, 595–599.
[15] Harlander, S. (1987) in Streptococcal Genetics (Ferretti, J.J. and Curtis III, R., eds.), pp. 229–233, Am. Soc. Microbiol., Washington, D.C.
[16] MacNeil, D.J. (1987) FEMS Microbiol. Lett. 42, 239–244.
[17] Chassy, B.M. and Flickinger, J.L. (1987) FEMS Microbiol. Lett. 44, 173–177.
[18] Powell, I.B., Achen, M.G., Hillier, A.J. and Davidson, B.E. (1988) Appl. Environ. Microbiol. 54, 655–660.
[19] Van der Lelie, D., van der Vossen, J.M.B.M. and Venema, G. (1988) Appl. Environ. Microbiol. 54, 865–871.
[20] Fiedler, S. and Wirth, R. (1988) Anal. Biochem. 170, 38–44.
[21] Kok, J., van der Vossen, J.M.B.M. and Venema, G. (1984) Appl. Environ. Microbiol. 48, 726–731.
[22] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523.
[23] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
[24] Golubkov, V.I., Gupalova, T.V., Kolesnichenko, T.G., Iontova, I.M., Malke, H. and Totolian, A.A. (1982) in Microbiology (Schlessinger, D., ed.), pp. 180–182, Am. Soc. Microbiol. Washington, D.C.
[25] Kok, J., Leenhouts, K.J., Haandrikman, A.J., Ledeboer, A.M. and Venema, G. (1988) Appl. Environ. Microbiol. 54, 231–238.
[26] Van der Vossen, J.M.B.M., Kok, J. and Venema, G. (1985) Appl. Environ. Microbiol. 50, 540–542.
[27] Vosman, B., Kooistra, J., Olijve, J. and Venema, G. (1987) Gene 52, 175–183.
[28] Golubkov, V.I., Reichardt, W., Boitsov, A.S., Iontova, I.M., Malke, H. and Totolian, A.A. (1982) Molec. Gen. Genet. 187, 310–316.

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Effect of growth conditions on the rate of loss of the plasmid pAT153 from continuous cultures of *Escherichia coli* HB101

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1. SUMMARY

The plasmid pAT153 was lost less rapidly from carbon, nitrogen, phosphorous or sulphur-limited continuous cultures of *Escherichia coli* HB101 as the dilution rate increased. At a fixed dilution rate of 0.3 h^{-1}, the plasmid was maintained longer as the growth-limiting nutrient was changed from glucose to casamino acids (nitrogen-limited), phosphate or sulphate. These differences in the stability of maintenance were not due to parallel changes in the plasmid copy number. We propose that the rate of loss of pAT153 from *E. coli* HB101 is determined primarily by the ratio of growth rates of plasmid-containing bacteria and plasmid-free bacteria. This ratio increases with increasing growth rate and depends markedly on the growth-limiting nutrient, sulphate-limited growth being particularly suitable for the maintenance of this host–plasmid combination.

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2. INTRODUCTION

Plasmid instability remains a potential problem in the use of recombinant plasmids to produce products of cloned genes on an industrial scale. In the absence of selection, plasmid-free segregants, which arise by mutations in replication, maintenance or partition functions, are at a selective advantage relative to the plasmid-bearing host.

Many improvements in plasmid stability have been achieved genetically [1–3]. In contrast, far less is known about how the relative selective advantage of plasmid-free segregants is influenced by the physiological status of the bacterial host. Wouters et al. [4] reported that the stability of maintenance of pBR322 in *Escherichia coli* increased with increasing growth rate in a carbon- or phosphate-limited chemostat. The same effect of growth rate on stability of pBR322 in *Klebsiella aerogenes* was found for glucose and ammonium limited cultures, but the stability of this plasmid decreased with increasing growth rate in a phosphate-limited culture [5]. Similarly, although pBR325 was more stable at high growth rates in carbon-limited cultures of *E. coli,* it was more stable at low growth rates in ammonium-limited cultures [6]. As conflicting trends have been re-

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ported even for the same host–plasmid combination (compare [7] with [8]) we have re-examined how the growth rate and growth limiting nutrient influence the stability of maintenance of the high copy number cloning vector pAT153 in continuous cultures of *E. coli* HB101.

In all of the previously reported studies of the stability of plasmid maintenance, continuous growth was limited by a nutrient that is required directly for plasmid replication as well as for host cell division. Although sulphate is equally required for the growth of plasmid-free and plasmid-bearing bacteria, it is only indirectly required in cysteine and methionine residues of proteins for plasmid replication. We have therefore also compared the rate of plasmid loss from glucose, ammonium and phosphate-limited cultures with that of a sulphate-limited culture.

3. MATERIALS AND METHODS

3.1. Bacterial strain, plasmid and growth conditions

The bacterial host, *Escherichia coli* HB101 (F^- *pro leu thi lacY rps*L31 *hsd*R *rec*A; [9]), was obtained from Searle Research and Development, High Wycombe, U.K. The plasmid, pAT153, carries the pMB1 replication origin. It is a derivative of pBR322 from which the 706 bp HaellB and G fragments were deleted in vitro. This deletion results in loss of the ROM protein and increases the plasmid copy number approximately 3-fold [10].

Cultures were grown in an MBR Minibioreactor with a working volume of 1 l, pH 7.0 ± 0.1, air supply 1 l min^{-1} and an agitation rate of 1000 rev. min^{-1} [11]. The semisynthetic growth medium contained 5 g l^{-1} glucose, 2.7 g l^{-1} casamino acids and 10 mg l^{-1} thiamine. Glucose and phosphate-limited cultures were supplemented with 7.94 g l^{-1} $(NH_4)_2SO_4$, which was replaced by 6.43 g l^{-1} NH_4Cl for sulphate-limited growth. The phosphorus source, 1.1 g l^{-1} KH_2PO_4, was decreased to 0.06 g l^{-1} for phosphate-limited growth. Casamino acids was the only source of sulphur for sulphate-limited cultures [11].

3.2. Screening for plasmid-containing bacteria

Bacteria to be screened for the ampicillin resistance determinant of pAT153 were plated onto starch agar and tested for secretion of β-lactamase activity during growth [12]. To confirm the presence of both ampicillin and tetracycline resistance determinants, colonies were replicated onto nutrient agar supplemented with either 100 μg ml^{-1} sodium ampicillin or 12.5 μg ml^{-1} tetracyline hydrochloride.

3.3. Detection of gross changes in plasmid structure and copy number

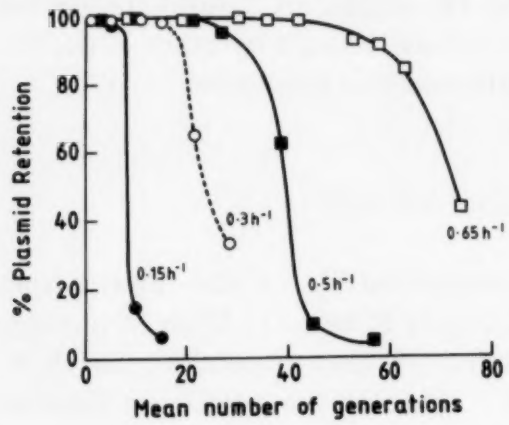
Plasmid DNA was isolated by the acid-phenol whole cell lysis method [13]. Bacterial cells equivalent to 1.0 ml of culture at an optical density at 650 nm of 2.0 were pelleted in a minifuge, washed in 0.5 ml sterile saline and resuspended gently in 100 μl of the lysis solution. The pBR322 standards were prepared simultaneously from a 16 h culture of *E. coli* HB101 (pBR322) which had been grown with vigorous aeration in 10 ml of L broth, 100 μg ml^{-1} disodium ampicillin and 12.5 μg ml^{-1} tetracycline hydrochloride. The extracted DNA samples (50 μl) were transferred to 10 μl of loading buffer (35% w/v Ficoll; 40 mM EDTA, 0.1% w/v bromophenol blue and 0.1% w/v xylene cyanol) and 1 μl of 1 mg ml^{-1} pancreatic ribonuclease. The samples were separated on a 1% (w/v) agarose gel which, after staining with 1 μg ml^{-1} ethidium bromide for 15 min, was photographed under ultraviolet light using Polaroid 665 positive-negative films. The negatives were scanned using a Joyce-Loebl Chromoscan 3 micro-densitometer to compare the densities of the plasmid bands with that of pBR322. Control experiments established that these densities were proportional to the quantity of plasmid DNA loaded onto the gel. Copy numbers were calculated on the assumption (which has been confirmed in hybridization experiments by M.E. Nugent; personal communication) that the copy number of pBR322 is 50 under the growth conditions used.

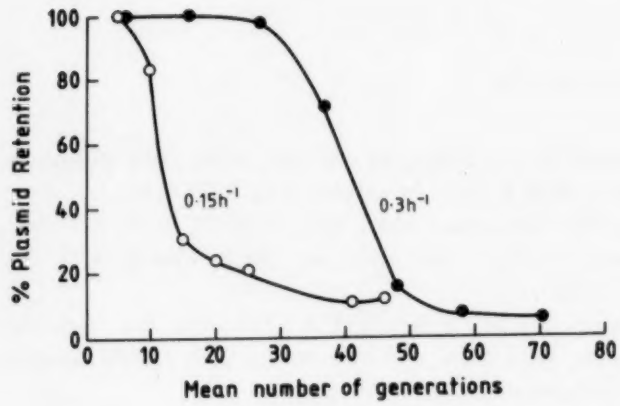
4. RESULTS

4.1. Effect of growth rate on plasmid maintenance

The effect of changes in the growth rate on plasmid stability was determined by growing *E. coli* HB101 (pAT153) in glucose-limited continu-

ous culture at dilution rates of 0.15 h^{-1}, 0.3 h^{-1}, 0.5 h^{-1} and 0.65 h^{-1} which correspond to mean generation times of 4.2 h, 2.31 h, 1.39 h and 1.07 h, respectively. Serial dilutions of samples taken at regular intervals were plated onto solidified media of composition similar to the feed medium. After 24 h at 37 °C, colonies were first tested for secretion of β-lactamase activity and then replica plated onto antibiotic-supplemented media of the same composition to check for retention of pAT153-encoded tetracycline and ampicillin resistance. In each experiment the fraction of plasmid-bearing cells decreased to a very low value, but the delay before a significant fraction of plasmid-free segregants was detected increased with increasing growth rate (Fig. 1a). Similar observations were made with both phosphate and sulphate-limited cultures (Fig. 1b, 1c). Note, however, that the plasmid was only lost at a significant rate from the sulphate limited cultures at a very low dilution rate of 0.06 h^{-1} which corresponds to a mean generation time of 11.55 h.





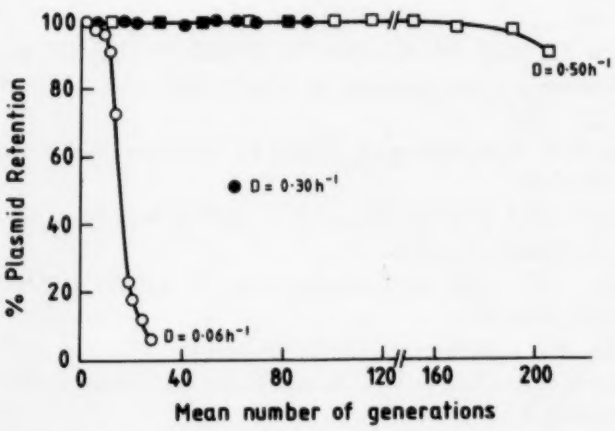


Fig. 1. Effect of growth rates on the rate of loss of the plasmid pAT153 from different nutrient-limited chemostat cultures of *E. coli* HB101. The growth-limiting nutrient was (a) glucose, (b) phosphate and (c) sulphate.

4.2. Effect of changing the growth-limiting nutrient on plasmid maintenance

At a fixed dilution rate of 0.3 h^{-1} the rate of plasmid loss depends on which nutrient limits growth. The plasmid had been lost by 10% of the population after 19 generations of growth in a glucose-limited culture (Fig. 1a), but was apparently more stable in ammonium or phosphate-limited cultures in which 10% of the population had lost the plasmid after 27 and 33 generations, respectively (unpublished result and Fig. 1b). In contrast, less than 1% of the bacteria had lost the plasmid after 90 generations of growth in the sulphate limited culture (Fig. 1c).

In a minority of the experiments, for example the ammonium-limited culture at $D = 0.3\ h^{-1}$, the fraction of plasmid-bearing bacteria stabilized or even increased after an initial decrease. Experiments to be described elsewhere established that this was caused by a mutation in the plasmid bearing population which allowed the mutant host to compete effectively with the plasmid-free segregants for the growth-limiting nutrient. The plasmid was also eventually lost from these cultures as plasmid-free segregants with an even greater selective advantage arose from the mutant population (see also reference [14]).

4.3. Effect of growth rate and growth limiting nutrient on plasmid copy number

If pAT153 is partitioned entirely randomly during cell division, differences in stability of maintenance during growth in different media might be due to differences in the mean plasmid copy num-

ber. Contrary to this expectation, the copy number remained constant, within experimental error, at about 160 per plasmid-containing cell. As there was no detectable increase in plasmid copy number with increased growth rate, the rate at which the plasmid-containing bacteria were lost from a chemostat must have been determined by other factors.

5. DISCUSSION

The chemostat experiments with *E. coli* HB101 (pAT153) confirmed that, in glucose-limited cultures, the plasmid was maintained longer with increasing growth rate. This effect was also detected when growth was limited by either phosphate or sulphate ions. At a fixed dilution rate of 0.30 h^{-1}, pAT153 was more stable in sulphate-limited cultures than in those limited by other nutrients.

The above observations could be explained by considering the two major factors which determine the rate of loss of plasmid-containing bacteria from a chemostat. These are the probability of segregation of plasmid-free bacteria and the selective advantage of these off-spring relative to their parents. Segregation of plasmid-free bacteria could in turn result from either defective plasmid replication or inaccurate partitioning. The probability that a plasmid-free cell would arise by chance when 100 copies of the plasmid are partitioned at random is infinitesimally small, 10^{-30} per cell per generation. The lack of a direct correlation between the average plasmid copy number and stability of maintenance during continuous growth is therefore not surprising. In contrast, there is a high probability that replication-defective mutants will arise spontaneously amongst the 10^{12} or more bacteria in each litre of chemostat culture.

The effect of growth rate on the rate of plasmid loss could reflect changes in the competitive advantage of plasmid-deficient bacteria in a mixed population relative to their plasmid-containing parents. For example, if the two strains have similar maximum specific growth rates but different saturation constants for the growth-limiting nutrient, the difference between their specific growth rates would be greater the lower the dilution rate.

Carbon, nitrogen and phosphorous are major components of nucleic acids. In contrast, sulphur is not a constituent of nucleic acids and it is only required indirectly as a minor component of the enzymes required for DNA synthesis. We therefore predicted that the stability of plasmid maintenance would be far greater in a sulphate-limited culture than in cultures limited by other nutrients. Our results confirmed this prediction.

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REFERENCES

[1] Caulcott, C.A., Lilley, G., Wright, E.M. and Robinson, M.K. (1985) J. Gen. Microbiol. 131, 3355–3365.
[2] Meacock, P.A. and Cohen, S.N. (1980) Cell 20, 529–542.
[2] Summers, D.K. and Sherratt, D.J. (1984) Cell 36, 1097–1103.
[4] Wouters, J.T.M., Driehuis, F.L., Polaczek, P.J., van Oppenracy, M.-L.H.A. and van Andel, J.G. (1980) Antonie van Leeuwenhoek 46, 353–362.
[5] Sterkenberg, A., Prozee, G.A.P., Leegwater, P.A.J. and Wouters, J.T.M. (1984) Antonie van Leeuwenhoek 50, 397–404.
[6] Noack, D., Roth, M., Geuther, R., Muller, G., Undisz, K., Hoffmeier, C. and Gaspar, S. (1981) Mol. Gen. Genet. 184, 121–124.
[7] Jones, S.A. and Melling, J. (1984) FEMS Microbiol. Lett. 22, 239–243.
[8] Dawson, M.J. and Lawrence, G.C. (1985) Soc. Gen. Microbiol. Quart. 12, M16.
[9] Boyer, H.W. and Roulland-Dussoix, D. (1969) J. Mol. Biol. 41, 459–472.
[10] Twigg, A.J. and Sherratt, D. (1980) Nature, 283, 216–218.
[11] Chew, L.C.K., Tacon, W.C.A. and Cole, J.A. (1986) FEMS Microbiol. Lett. 36, 275–280.
[12] Boyko, W.L. and Ganschow, R.E. (1982) Anal. Biochem. 122, 85–88.
[13] Kado, C.I. and Liu, S.T. (1981) J. Bacteriol. 145, 1365–1373.
[14] Helling, R.B., Kinney, T. and Adams, J. (1981) J. Gen. Microbiol. 123, 129–141.

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Effect of nicking on Shiga-like toxin I of enterohaemorrhagic *Escherichia coli*

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Key words: Vero toxin; Nicked and unnicked Shiga-like toxin; Cytotoxic activity

1. SUMMARY

The Shiga-like toxin I (SLT-I), which had an unnicked A subunit, was purified from *Escherichia coli* O157 : H7. After incubation of the unnicked SLT-I with trypsin, the A subunit of the toxin was nicked and converted to A_1 and A_2 peptides, which were linked by the disulfide bond. The effects of nicking of SLT-I on its heat stability and cytotoxic activity against Vero cells were examined. The trypsin treatment of SLT-I neither reduced nor increased the ability of cytotoxic activity or the heat stability of the toxin. The time course of binding of the toxin to Vero cells also was not affected by nicking of the toxin. These results indicate the absence of a difference in biological or physical properties of SLTs between nicked and unnicked conditions.

2. INTRODUCTION

Escherichia coli O157 : H7, an etiologic agent of haemorrhagic colitis [1,2], produces two related cytotoxins namely Shiga-like toxin I and II (SLT-I and SLT-II). Although SLT-II has unique immunogenicity, SLT-I was found to be structurally and immunologically indistinguishable from Shiga toxin produced by *Shigella dysenteriae* [3,4]. SLT-I consists of A and B subunits [4]. The B subunit is responsible for the binding of toxin to the target cell receptor, whereas the A subunit is the component responsible for inhibiting protein synthesis in the target cell [4,5]. The biologically active A subunit is composed of two peptides, A_1 and A_2 derived from the A subunit by a proteolytic cleavage.

Studies on heat-labile enterotoxin (LT) produced by enterotoxigenic *E. coli* [6,7] and *Clostridium botulinum* toxin [8] have shown that the toxin nicked by trypsin has a higher activity than the unnicked toxin. LT nicked by trypsin has been reported to induce a morphological change of chinese hamster ovary (CHO) cells faster than unnicked LT [9]. However, the effect of nicking of SLT-I on its cytotoxic activity and heat stability has not been studied in detail yet.

We recently developed a simple method for purification of SLT-I [10], and the purified SLT-I by this method was usually unnicked. Thus, it is possible to compare the properties of nicked and unnicked SLT-I.

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In this paper we studied the detailed effects of nicking of SLT-I on its heat stability and cytotoxic activity against Vero cells and found no difference in cytotoxic activity or heat stability between nicked and unnicked SLTs.

3. MATERIALS AND METHODS

3.1. Preparation of SLT-I

SLT-I was purified from an *E. coli* O157 : H7 strain used previously [10]. The culture conditions, preparation of crude toxin, and purification of SLT-I by two steps of column chromatographies were described in detail elsewhere [10]. The purified SLT-I gave two bands corresponding to the A and B subunits on sodium dodecyl sulfate-polyacrylamide gel slab electrophoresis (Fig. 1).

3.2. Treatment of SLT-I with trypsin

The purified toxin (20 μg) in 0.2 ml of 50 mM ammonium bicarbonate (pH 8.3) was incubated with 0.5 μg of trypsin (Sigma Chemical Co., St. Louis, MO., U.S.A., Type III from bovine pancreas) at 37°C for 1 h. The cytotoxic activity of the toxins treated with and without trypsin were compared immediately after trypsin treatment. The test samples were also heated at 60°C for 10 min or 100°C for 5 min and their cytotoxicity was determined to examine the effect of nicking on heat stability of the toxins.

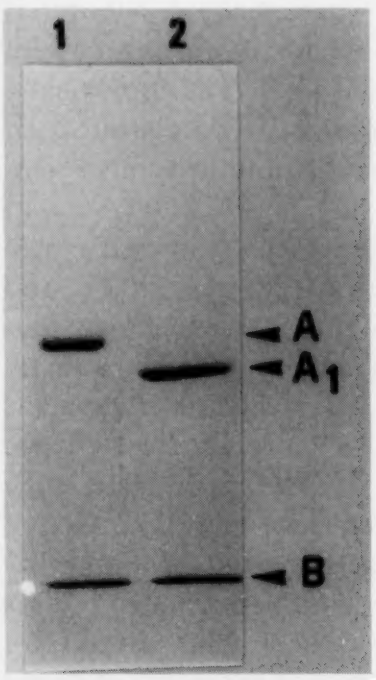

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel slab electrophoresis of SLT with (nicked SLT) and without (unnicked SLT) trypsin treatment. Lane 1, unnicked SLT; lane 2, nicked SLT. A, A subunit; A_1, A_1 peptide; B, B subunit.

3.3. Assay for cytotoxic activity

Cytotoxic activity of SLT-I was determined by using Vero cells cultured in 96-well microtiter plates. A test sample (30 μl) was added to each well and then sterilized by exposing the plate to UV light for 30 min. Then about 5×10^3 Vero cells suspended in 200 μl of Eagle minimum essential medium (MEM) supplemented with 10% calf serum and gentamicin (50 μg/ml) was added to each well of the microtiter plates [11,12]. The plates were further incubated at 37°C in a 5% CO_2 atmosphere for 24 h. The change of morphology was observed under a phase contrast microscope.

3.4. Immunofluorescence staining

The nicked and unnicked SLTs (0.5–5.0 μg/ml) were challenged to Vero cells grown in 8-chamber tissue culture slides (Lab-Tek Products, Naperville, IL). The slides were incubated at 37°C under 5% CO_2 atmosphere for the indicated time, then washed with phosphate buffered saline (PBS, pH 7.2) and fixed with 8% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) containing 8% sucrose for 10 min. The fixed cells were washed with PBS and incubated in the presence of 0.1 ml of purified anti-Shiga toxin immunoglobulin dissolved in PBS at 37°C for another 1 h in a humidified chamber. The cells were washed again and the fluorescein conjugated IgG fraction of goat anti-rabbit IgG (Cooper Biomedical Scientific Division, West Chester, PA) diluted (1 : 400) in PBS was added. After incubation at 37°C for 30 min and thorough washing with PBS, the cells were examined and automatically photographed (Microflex Nikon UFX) under an incident fluorescence microscope (Fluophot Nikon Microphot V series, Nikon).

4. RESULTS

4.1. Trypsin treatment of SLT-I

After treatment of SLT-I with trypsin as described in MATERIALS AND METHODS, the toxin was proteolytically nicked (nicked SLT-I) and the A subunit was converted to form A_1 and A_2 peptides, which migrated faster than the A subunit (Fig. 1).

4.2. Cytotoxic activity of nicked and unnicked SLT-I

The nicked and unnicked SLTs thus obtained were compared for cytotoxic activity by incubation of the toxins with Vero cells. The cytotoxic activity of nicked SLT-I was similar to that of unnicked SLT-I. The amount of toxin causing 100% cytotoxic dose (CD_{100}) of both nicked and unnicked SLT-I was determined as 0.1 ng/ml.

4.3. Heat stability of nicked and unnicked SLTs

To determine the heat stability of nicked and unnicked SLTs, both types of toxin were heated at 60 °C for 10 min and 100 °C for 5 min, and then the cytotoxic activity of heat-treated toxins was compared (Table 1). We found no difference in cytotoxic activity of the toxins, suggesting that the heat stability of nicked and unnicked SLTs were the same.

4.4. Binding time to Vero cells of nicked and unnicked SLT-I

Vero cells grown in the 96-well microplates were incubated with nicked and unnicked SLTs for the indicated times. After removal of the toxin by washing, the cells were further incubated and the cytotoxic activity was observed 24 h later. We found that as with the nicked SLT-I, the unnicked SLT-I showed a cytotoxic effect by incubation of Vero cells with the toxins for 5 min (Table 2), indicating that incubation for 5 min is enough for binding of both nicked and unnicked SLTs to Vero cells.

The time required for binding of nicked and unnicked SLTs to Vero cells was also examined by the immunofluorescence staining technique. Vero cells grown in the chamber slides were incubated with nicked and unnicked SLT-I for the indicated times. Then, the toxin was washed out and the Vero cells were stained by the immunofluorescence technique (Table 3). The positive staining of nicked and unnicked SLT-I was detected after their incubation with the cells for 5 min. The data

Table 1

Heat stability and cytotoxic activity of nicked and unnicked SLTs

Conditions	SLT-I	
	Nicked	Unnicked
Unheated	0.2 ng [a]	0.2 ng
60 °C, 10 min	0.2 ng	0.2 ng
100 °C, 5 min	1.0 μg	1.0 μg

[a] Minimum amount of toxin necessary.

Table 2

Effect of incubation time on cytotoxic activity of nicked and unnicked SLTs

Incubation time [a] (min)	Cytotoxic activity	
	Nicked SLT-I	Unnicked SLT-I
2	–	–
5	+	+
15	+	+
30	+	+
60	+	+
120	+	+

[a] Vero cells grown in 96-well microplates were incubated with nicked and unnicked SLT (10 CD_{100}) for the indicated times. Then the toxin was washed off, and the cells were incubated further for 24 h before examination.

Table 3

Binding time of nicked and unnicked SLT-I to Vero cells examined by immunofluorescence technique

Incubation time (min)	Immunofluorescence staining					
	Nicked SLT-I			Unnicked SLT-I		
	0.5 μg	2 μg	5 μg	0.5 μg	2 μg	5 μg
5 [a]	–	+	+	–	+	+
10	+	+	+	+	+	+
30	+	+	+	+	+	+
60	+	+	+	+	+	+
180	+	+	+	+	+	+

[a] Vero cells grown in chamber slides were incubated with nicked and unnicked SLTs for the indicated times. After unbound toxin was washed away, the cells were fixed and stained.

also indicate no difference in binding time of nicked and unnicked SLT-I to Vero cells.

5. DISCUSSION

Trypsin has been used to activate various toxins such as unnicked *E. coli* LT enterotoxin [6,7] and *C. botulinum* toxin [8]. The nicking *E. coli* LT with trypsin also affected the time course of the activity of LT on CHO cells [9]. However, it was reported that unnicked Shiga toxin produced by *S. dysenteriae* inhibited protein synthesis in HeLa S_3 cells to the same extent as nicked toxin, at least under the limited experimental conditions (incubation at 37°C overnight) [13].

The present study reports the effect of nicking of SLT-I on the biological or physical properties of the toxin. The results show that, when unnicked SLT-I was incubated with trypsin, like Shiga toxin [13], the A subunit of SLT-I was converted to A_1 fragment as demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, but unlike LT, the heat stability and cytotoxic activity of SLT-I were neither reduced nor increased by trypsin treatment. The properties of nicked SLT-I, such as cytotoxic activity, heat stability, and time necessary for binding to Vero cells, are similar to those of unnicked SLT-I. These results suggest that nicking in the A subunit of SLT-I did not affect the biological or physical properties of SLT-I.

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REFERENCES

[1] Johnson, W.M. and Bezanson, G.S. (1983) Lancet i, 76.
[2] O'Brien, A.D., Lively, T.A., Chang, T.W. and Gorbach, S.L. (1983) Lancet ii, 573.
[3] O'Brien, A.D. and Laveck, G.D. (1983) Infect. Immun. 40, 675–683.
[4] O'Brien, A.D. and Holmes, R.K. (1987) Microb. Rev. 51, 206–220.
[5] Reisbig, R., Olsnes, S. and Eiklid, K. (1981) J. Biol. Chem 8739–8744.
[6] Donta, S.T., Poindexter, N.J. and Ginsberg, B.H. (1982) Biochem. 21, 660–664.
[7] Rappaport, R.S., Sagin, J.F., Pierzchala, W.A., Bonde, G., Rubin, B.A. and Tint, H. (1976) J. Infect. Dis. 133 (Suppl.), 41–45.
[8] Duff, J.T., Wright, G.G. and Yarinsky, A. (1956) J. Bacteriol. 72, 455–460.
[9] Tsuji, T., Honda, T. and Miwatani, T. (1984) Infect. Immun. 46, 94–97.
[10] Kongmuang, U., Honda, T. and Miwatani, T. (1987) FEMS Microbiol. Lett. 48, 379–383.
[11] Gentry, M.K. and Dalrymple, J.M. (1980) J. Clin. Microbiol. 12, 361–366.
[12] Kongmuang, U., Honda, T. and Miwatani, T. (1987) J. Clin. Microbiol. 25, 115–118.
[13] Olsnes, S., Reisbig, R. and Eiklid, K. (1981) J. Biol. Chem. 256, 8732–8738.

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Haemagglutinating activity of *Campylobacter pylori*

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1. SUMMARY

Thirty-one isolates of *Campylobacter pylori*, screened for their ability to agglutinate a panel of erythrocyte species, could be divided into two phenotypic groups on the basis of their ability to agglutinate human A and O erythrocytes, a property which correlated strongly with their ability to agglutinate horse and cat erythrocytes. Isolates which agglutinated human red blood cells exhibited a broad-spectrum haemagglutination profile on other red blood cells including dog, goat, guinea-pig, ox, rat and sheep erythrocytes. Agglutination of dog, guinea-pig, horse and human erythrocytes by *C. pylori* was mannose-resistant. Haemagglutination was not inhibited by other saccharides tested nor by two glycoproteins or serine. The bacterial ligand was protease- and heat-sensitive. Neither protease nor neuraminidase treatment of erythrocytes prevented agglutination.

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2. INTRODUCTION

The strong association between duodenal ulceration, antral gastritis and antral colonisation with spiral-shaped bacteria has stimulated interest in the potential role(s) of these bacteria in the pathogenesis of gastroduodenitis [1–6]. The bacterium is presently called *C. pylori* (previously *C. pyloridis*) [7], although its taxonomic status remains uncertain [8].

A virulence attribute common to intestinal bacterial pathogens is the ability to adhere to epithelial cells [9–12]. Many of the adhesins involved in such bacteria–host cell interactions enable the bacteria to agglutinate erythrocytes of different species [12–14]. Haemagglutination has provided a useful means for the classification of bacterial adhesins and a model system for the investigation of ligand-receptor interactions at the molecular level [9,10,15]. In many such interactions, bacterial fimbriae act as the ligands [9–15].

The present studies were undertaken to investigate (i) the ability of *C. pylori* to agglutinate erythrocytes, (ii) the sugar specificity(ies) of any haemagglutination reactions, and (iii) the effects of protease, neuraminidase and heat treatments on bacteria-erythrocyte interactions.

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3. MATERIALS AND METHODS

3.1. Isolation and identification of C. pylori *strains*

Antral biopsies, obtained from dyspeptic patients attending an endoscopy clinic, were inoculated directly onto selective Campylobacter agar [Campylobacter Agar Base (Oxoid) containing Skirrow Selective Supplement (Oxoid) and 7% (v/v) lysed horse blood (Tissue Culture Services, Slough, U.K.)]. Plates were incubated at 37°C for 48–72 h in an anaerobic jar (Oxoid) using a Gas Generating Kit (Oxoid) for campylobacters and microaerophilic organisms. Typical, small (circa 1 mm diameter), smooth, transparent colonies were Gram-stained and subcultured onto lysed horse blood Campylobacter (LBC) agar (without selective supplement). Isolates were identified as *C. pylori* on the basis of colony morphology, typical Gram-stain morphology, and catalase and urease tests [16,17]. The isolates were preserved at −70°C in Tryptone soya broth (Oxoid) containing 15% (v/v) glycerol or in 60% (v/v) lysed horse blood: 40% (v/v) peptone water. The thirty-one isolates used in the present studies were obtained from 31 individuals.

3.2. Haemagglutination tests

Citrated cat, dog, goat, guinea-pig, horse, ox, pig, rabbit, rat and sheep blood were obtained fresh. Human A and O blood were outdated HIV-negative samples from the local blood bank.

Red blood cells (RBCs) were washed two to three times in physiological saline and prepared as 3% (v/v) suspensions of packed cell volume in phosphate buffered saline [PBS; 0.145 M NaCl containing 10 mM sodium phosphate buffer, pH 7.2]. LBC agar-grown (37°C, 48 h) bacteria were suspended in PBS using a cotton-swab to circa 5×10^{10} bacteria/ml.

Two-fold doubling dilutions in PBS of bacterial suspensions (50 μl) were made in round-bottomed 96-well trays (Linbro). To each well was added 50 μl of RBC suspension. The tray was agitated to ensure even mixing, covered, incubated at room temperature (18°C) for 1 h, read, and allowed to settle overnight before final scoring according to a scale used for *Treponema pallidum* haemagglutination tests (Wellcome Laboratories).

The following solutions were prepared in PBS for use in haemagglutination inhibition tests: D-glucose, D-galactose, D-mannose, D-fucose, L-fucose, D-galactosamine, D-glucosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and α-lactose (all 100 mM); *N*-acetylneuramin-lactose (5 mg/ml); serine (20 mg/ml), dog α_1-acid glycoprotein, human α_1-acid glycoprotein (orosomucoid preparations; 2 mg/ml).

Each well in the inhibition assays contained 25 μl of a two-fold dilution of bacterial suspension, 25 μl of test inhibitor and 25 μl of erythrocyte suspension. Controls contained 25 μl of PBS instead of test inhibitor. Titrations were incubated and read as described above. Tests were performed in parallel with dog, goat, horse and human A RBC suspensions with each of three *C. pylori* isolates.

3.3. Treatment of red blood cells and bacteria prior to haemagglutination

RBC suspensions [3% (v/v) in PBS] were treated separately with trypsin, α- and β-chymotrypsin, protease type XIV (pronase), and *Clostridium perfringens* type X neuraminidase (all obtained from Sigma). Erythrocytes were incubated for 2 h at 37°C with 1 mg protease/ml, or for 1 h at 37°C with 0.1 unit neuraminidase/ml. The red blood cells were sedimented, washed twice with PBS and resuspended to the original volume. Using PBS instead of enzyme, control RBC suspensions were similarly processed. Titrations of three *C. pylori* isolates were tested in parallel with treated and control suspensions of erythrocytes.

Six isolates of *C. pylori* (ca. 5×10^{10} bacteria/ml in PBS) were treated separately with proteases as described above. Bacterial suspensions were also heated at 60°C, 80°C and 100°C for 1 h, sedimented and resuspended to the original volume. Control suspensions were all incubated at 37°C and room temperature, respectively, and processed identically to treated bacterial suspensions.

4. RESULTS

4.1. Haemagglutination

Initially 18 isolates of *C. pylori* were each

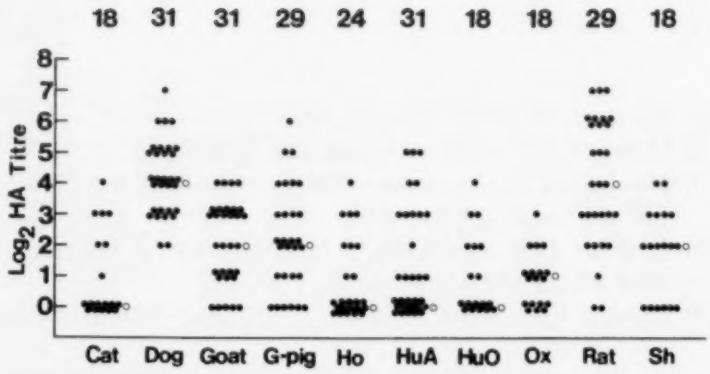

Fig. 1. Haemagglutination (HA) titres of *C. pylori* strains for a variety of erythrocyte species. All red blood cell species were tested initially with the same eighteen strains of *C. pylori*. Subsequently up to a further 13 strains were tested only on dog, goat, guinea-pig, horse, human A and rat erythrocytes. Titres for individual strains are indicated by the closed circles. The open circles indicate the median titres for the nos. of strains tested on each red blood cell species which are shown above the titre distributions. Erythrocyte species: Ho, horse; HuA, human blood group A; HuO, human blood group O; Sh, sheep.

screened against a panel of 10 erythrocyte species (Fig. 1). All of these isolates agglutinated both dog and rat erythrocytes with median titres of 1 : 32.

Two phenotypic groups emerged. Phenotype 1 isolates agglutinated human blood group A and O RBCs, whereas phenotype 2 isolates did not. Eight isolates belonged to phenotype 1. All eight isolates agglutinated goat, guinea-pig and sheep red blood cells and seven of them, ox red blood cells; six also agglutinated both cat and horse erythrocytes. The isolate which did not agglutinate ox erythrocytes failed to agglutinate both cat and horse erythrocytes. All the phenotype 2 isolates failed to agglutinate cat and horse erythrocytes. This phenotypic group showed five different haemagglutination patterns with respect to their ability to agglutinate goat, guinea-pig, ox and sheep erythrocytes (Table 1).

On the basis of these findings up to a further thirteen isolates were tested against a limited range of erythrocyte species—dog, goat, guinea-pig, horse, human A and rat red blood cells. The findings mirrored those with the original 18 strains (Fig. 1).

Rabbit and porcine red blood cells were excluded from these studies when it was discovered that surface washes of uninoculated LBC agar

Table 1

Haemagglutination patterns of ten phenotype 2 strains

C. pylori isolate no.	Agglutination of indicated RBC species			
	Goat	Guinea-pig	Ox	Sheep
187114	+	+	+	+
164912, 172488, 181798	+	+	−	+
115782, 134498, 162665	+	−	+	−
190896	+	−	−	−
184858, 191984	−	−	−	−

plates with a moistened swab contained an agglutinin for these erythrocyte species. The source of this agglutinin was traced to the Oxoid agar component of the Campylobacter agar base. None of the other ten erythrocyte species initially tested (Fig. 1) reacted with this agglutinin.

4.2. Haemagglutination inhibition assays and the effects of pretreatments of bacteria or RBCs on haemagglutination

Mannose did not inhibit agglutination of dog, guinea-pig, horse and human A erythrocytes. None of the other 10 saccharides tested inhibited these haemagglutination reactions. Tests with dog α_1-acid glycoprotein, human α_1-acid glycoprotein and serine were similarly negative at the concentrations tested.

Dog, goat, horse and human A erythrocytes treated with either trypsin, α- or β-chymotrypsin or pronase were agglutinated by the three phenotype 1 isolates and one phenotype 2 isolate tested with one exception. Trypsin treatment of goat erythrocytes rendered them autoagglutinable, thereby negating this particular test. Neuraminidase treatment of these four erythrocyte species did not affect their agglutinability.

Treatment of four phenotype 1 and two phenotype 2 isolates of *C. pylori* with either trypsin, α- or β-chymotrypsin or pronase abolished the haemagglutinating activity of these isolates against dog, goat, horse and human A erythrocytes (haemagglutination titres ranging from 1 : 4 to 1 : 32 were reduced to $< 1 : 2$).

Heating suspensions of three phenotype 1 isolates of *C. pylori* at 60 °C reduced haemagg-

lutinating activity by 50–90%, whereas treatment at 80 °C and 100 °C abolished haemagglutinating activity completely for dog, goat, horse and human A erythrocytes.

5. DISCUSSION

The haemagglutination properties of *C. pylori* have not been previously described. Grouping of isolates into haemagglutination phenotypes may prove useful in future epidemiological studies. Recent evidence suggests that different strains of *C. pylori,* identified by immunofingerprinting, may not be equally virulent as judged by the clinical correlations and the degree of histological gastritis [18]. Phenotypic differences in haemagglutination activity between strains may also manifest differences in virulence.

The ligand on *C. pylori* involved in haemagglutination was shown to be both heat- and protease-sensitive. This implicates a protein as ligand. Investigations are presently underway to identify the morphological nature of the ligand on the bacterial cell surface by electron microscopy. The haemagglutination reactions with four erythrocyte species were not inhibited by several saccharides commonly used as receptor antagonists [10,13,15]. However, *C. pylori* exhibited mannose-resistant haemagglutination, a property which has been associated with the presence of specific ligand–receptor interactions of many bacteria. The present studies were performed with bacteria grown on LBC agar. Cultural conditions may affect the expression of the haemagglutinin(s). Further studies are required to define optimal culture conditions for haemagglutinin expression and to determine whether or not the haemagglutinin is an adhesin involved in attachment of *C. pylori* to the gastroduodenal epithelium.

REFERENCES

[1] Marshall, B. (1983) Lancet i, 1273–1275.
[2] Marshall, B.J. (1986) J. Infect. Dis. 153, 650–657.
[3] Goodwin, C.S., Armstrong, J.A. and Marshall, B.J. (1986) J. Clin. Pathol. 39, 353–365.
[4] Graham, D.Y. and Klein, P.D. (1987) Am. J. Gastroenterol. 82, 283–286.
[5] Dooley, C.P. and Cohen, H. (1988) Ann. Intern. Med. 108, 70–79.
[6] Lee, A. and Hazell, S.L. (1988) Microb. Ecol. Health Dis. 1, 1–16.
[7] Marshall, B.J. and Goodwin, C.S. (1987) Int. J. System. Bacteriol. 37, 68.
[8] Romaniuk, P.J., Zoltowska, B., Trust, T.J., Lane, D.J., Olsen, G.J., Pace, N.R. and Stahl, D.A. (1987) J. Bacteriol. 169, 2137–2141.
[9] Mirelman, D. (1986) Microbial Lectins and Agglutinins: Properties and Biological Activity, John Wiley and Sons Inc., New York, 443 pp.
[10] Lark, D.L., Normark, S., Uhlin, B.-E. and Wolf-Watz, H. (1986) Protein-Carbohydrate Interactions in Biological Systems, FEMS Symposium No. 31, Academic Press, London, 464 pp.
[11] Old, D.C. (1985) Med. Lab. Sci. 42, 78–85.
[12] Jones, G.W. and Isaacson, R.E. (1984) C.R.C. Crit. Rev. Microbiol. 10, 229–260.
[13] Smyth, C.J. (1986) in Antigenic Variation in Infectious Diseases (Birkbeck, T.H. and Penn, C.W., eds.), Special Publications of the Society for General Microbiology No. 19, pp. 95–125, IRL Press, Oxford.
[14] Old, D.C. (1985) in The Virulence of *Escherichia coli*: Reviews and Methods (Sussman, M., ed.), Special Publications of the Society for General Microbiology No. 13, pp. 287–313, Academic Press, London.
[15] Wadström, T. and Trust, T.J. (1984) in Medical Microbiology (Easmon, C.S.F. and Jeljaszewicz, J., eds.), Vol. 4, pp. 287–334, Academic Press, London.
[16] McNulty, C.A.M. and Dent, J.C. (1987) J. Clin. Microbiol. 25, 1683–1686.
[17] Hazell, S.L., Borody, T.J., Gal, A. and Lee, A. (1987) Am. J. Gastroenterol. 82, 292–296.
[18] Burnie, J., Lee, W., McNulty, C. and Dent, J. (1988) Lancet i, 302.

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Comparative study on the cell wall structure of protein-producing *Bacillus brevis*

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1. SUMMARY

Among eight strains of protein-producing *Bacillus brevis*, three morphological groups have been identified according to the structure of the cell walls.

(I) Cell wall consisting of a peptidoglycan-layer
(II) Two-layered cell wall consisting of a peptidoglycan-layer and an S-layer
(III) Three-layered cell wall consisting of a peptidoglycan-layer and two S-layers

Group I and group II cell walls have not been described yet for protein-producing bacteria. The S-layers observed in this study all had hexagonal symmetry and lattice constants of approximately 18 nm. The immunological relation between the S-layer proteins of the newly isolated *B. brevis* strains and those of *B. brevis* 47 has been examined using antisera against both S-layer-proteins of *B. brevis* 47. S-layers from protein-producing *B. brevis* strains, which were adjacent to the peptidoglycan-layer, were similar to each other, whether they were the outermost cell wall layer (group II) or not (group III). However, no similarity was found between these layers and the outermost S-layer of *B. brevis* 47 (group III).

2. INTRODUCTION

In the course of a screening program for protein-producing bacteria, many of the strains have been identified as *Bacillus brevis* sp. Among *B. brevis* the protein-producing strain 47 has been biochemically and genetically characterized [1–3]. Strain 47 possesses a three-layered cell wall consisting of a peptidoglycan-layer and two protein-layers (S-layers), termed the middle wall (MW) and outer wall (OW). They are serologically different and the main constituents of the secreted protein [4]. Out of the recently isolated *B. brevis* strains, eight strains have been chosen for analysis of their cell wall structures to examine whether there exists a common feature in cell wall morphology. Therefore, negative staining followed by optical diffraction as well as ultrathin sectioning was performed with cell wall preparations and whole cells, respectively. The immunological relation between the new isolates and *B. brevis* 47 was tested by Western blot analysis.

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3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

Bacillus brevis strains HP019, HP020, HP033, HP034, HPD31, HPD52, HPD64 and HPD71 were grown at 37°C in 50 ml of T2-medium as previously described [5]. All strains were supplied by K. Kadowaki and H. Takagi (Higeta Shoyu, Co. Ltd.). Isolation and identification of strains used here will be published elsewhere.

3.2. Preparation of cells for electron microscopy

Thin sectioning of whole cells was performed as described [5]. For negative staining of purified cell wall preparations (drop floating method) grids coated with Formvar were used. The samples were stained with 1% uranyl acetate in destilled water for 1 min and air dried. Cell walls were prepared from mid-log-phase cultures as follows. The cells were harvested by centrifugation (7000 × *g*, 15 min, 4°C) and washed once with 50 mM TRIS-HCl buffer, pH 7.5. The pellet from 800 ml culture was suspended in 20 ml of TRIS-HCl buffer, pH 7.5, containing 10 mM $MgSO_4 \cdot 7H_2O$, mixed with an equal volume of glass beads (0.1 to 0.11 mm) and broken four times in 30 s intervals in a Braun homogenizer fitted with a cooling device. After removing unbroken cells (5000 × *g*, 10 min, 4°C), the envelope fraction was centrifuged (25 000 × *g*, 20 min, 4°C) and washed once in the Mg^{2+}-containing buffer. The cell membrane was extracted by 60 min incubation with gentle shaking at 37°C in 5 ml of the same buffer containing 2% Triton X-100. Then the cell walls were centrifuged (25 000 × *g*, 20 min, 4°C) and washed three times in the Mg^{2+}-containing buffer. When resuspending the cell walls during the washing procedure, care was taken to remove only the upper layer, i.e. the pure cell wall fraction. The pellets were stored in the same buffer at 4°C. The preparations were examined under a JEOL JEM-100CX electron microscope at an acceleration voltage of 80 kV.

3.3. Optical diffraction

Optical diffraction was performed using a Nihon Bunko Ltd. diffractometer of standard design with Tropel lenses and a Spectra-Physics helium-neon laser as light source.

3.4. Analysis of cell wall proteins

Sodium dodecylsulfate-polyacrylamide gel (10%) electrophoresis (SDS-PAGE) was performed as described in [6], followed by either staining with Coomassie Brilliant Blue R-250 or electrophoretical transfer to nitrocellulose sheets for detection of immunoreactive protein bands as described in [7]. To visualize proteins cross-reactive with the antibodies to either the OW- or MW-protein of *B. brevis* 47, horseradish peroxidase-conjugated second antibody and the chromophore 4-chloro-1-naphthol were used as described in [3].

4. RESULTS

4.1. Cell wall protein composition of B. brevis *strains and their immunological relation*

When crude cell wall fractions prepared from the eight strains introduced here were analyzed for their protein-composition by SDS-PAGE, three strains, HPD31, HPD52 and HP033 had major cell wall proteins at a high molecular weight range. The other five strains, HP019, HP020, HP034, HPD61 and HPD71 did not contain such proteins (data not shown). For further analysis, we selected four strains, three containing major cell wall proteins and one, HP034, as a representative strain lacking a major cell wall protein. Fig. 1A shows the protein composition of cell wall fractions purified from the four strains described above. The apparent molecular masses of the major cell wall proteins of strain HPD31 were 135 kDa with a minor band at 150 kDa. Also strain HPD52 had an additional band (75 kDa) to the most prominent band at 115 kDa. Strain HP033 contained two major cell wall proteins with apparent molecular masses of 110 and 150 kDa. No protein bands were observed with strain HP034. Fig. 1B shows the immunological relation of the cell wall proteins of the four strains with the MW-protein of strain 47. The antibody to the MW-protein of strain 47 cross-reacted with cell wall proteins of HPD31 (135 kDa), HPD52 (115 kDa) and HP033

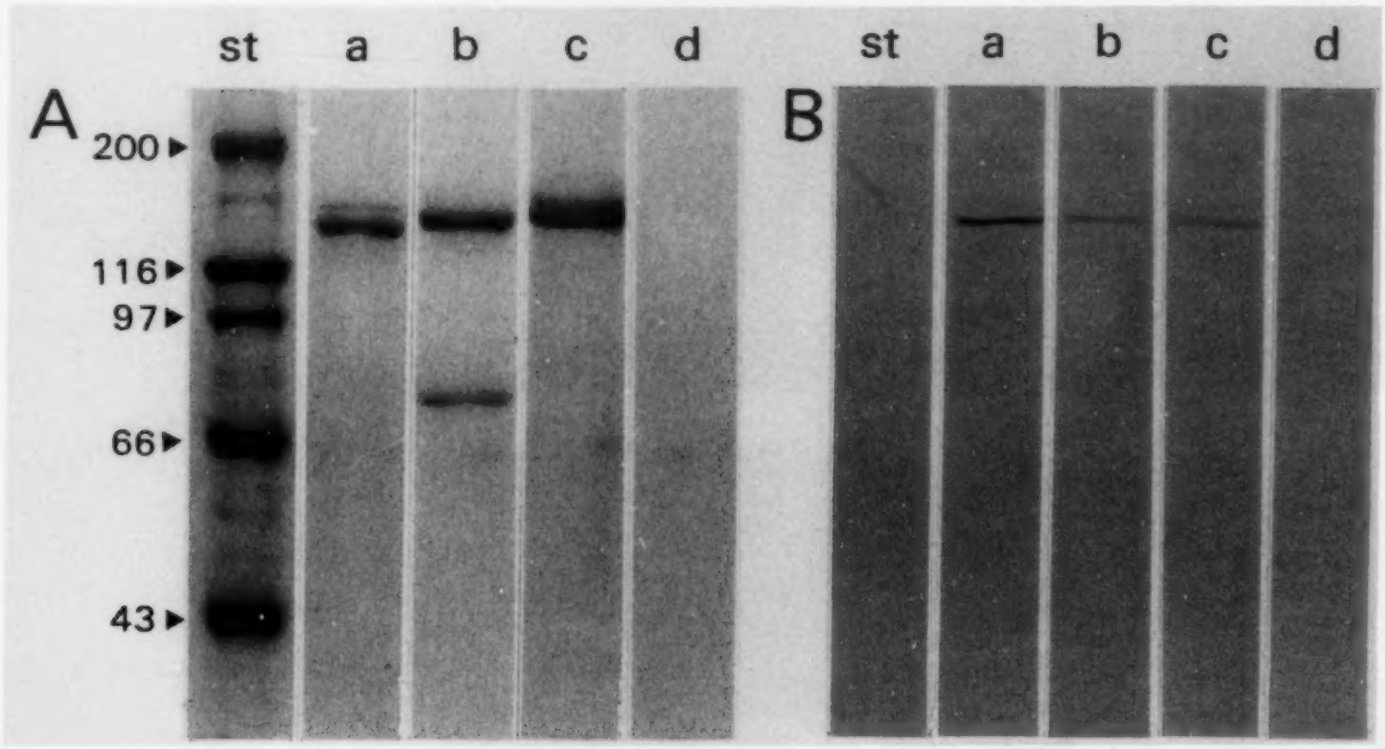

Fig. 1. SDS-polyacrylamide gel electrophoresis (A) and Western blot analysis (B) of cell wall proteins. *B. brevis* strain HPD31(a), HPD52(b), HP033(c) and HP034(d) cell wall proteins were electrophoresed together with the Bio-Rad standard proteins (st). For immunoblotting analysis, *B. brevis* 47 MWP-antiserum was used as described in the text.

(110 kDa). The antiserum against the OW-protein of strain 47 did not cross-react with any of the cell wall proteins of the strains under investigation (data not shown).

4.2. Negative stainings and optical diffractograms of the cell envelope preparations

Negatively stained preparations of the cell wall fractions of three strains, HPD31, HPD52 and HP033 (Fig. 2c–4c) showed a highly ordered surface structure (S-layer). With all three strains, the S-layer had hexagonal symmetry. The lattice constants calculated on the basis of the optical diffractograms (Fig. 2d–4d) were 18.1 nm for strain HPD31, 17.4 nm for HPD52 and 18.4 nm for HP033. No regular surface structure was observed with the cell walls of the other five strains. The micrograph of strain HP034 is shown in Fig. 5c with the corresponding optical diffractogram in Fig. 5d.

4.3. Structure of the cell walls as viewed in ultrathin-sectioned preparations of whole cells

Fig. 2a–5a show the cells in survey, Fig. 2b–5b the cell walls at higher magnification. *B. brevis* HPD31 and HPD52 (Fig. 2, 3) have a similar cell wall structure with a peptidoglycan layer approximately 2 nm strong and an S-layer of approximately 5 nm. Strain HP033 (Fig. 4a, b) has a more complex cell wall with two S-layers, termed the middle wall (MW) (7 nm) and the outer wall (OW) (4 nm), in addition to the peptidoglycan layer, which is approximately 2 nm strong. With strain HP034, the cell wall consists of an 8 to 9 nm thick peptidoglycan layer (Fig. 5a, b).

DISCUSSION

A remarkable heterogeneity in the protein composition and the cell wall structure was found within eight strains of protein-producing *Bacillus brevis.* Three groups were introduced according to the data obtained.

The cell wall of group I bacteria is a peptidoglycan-layer, appearing as sacculi without a structured surface in negatively stained cell wall preparations (Fig. 5c). Correspondingly no protein bands were observed when extracts of purified cell

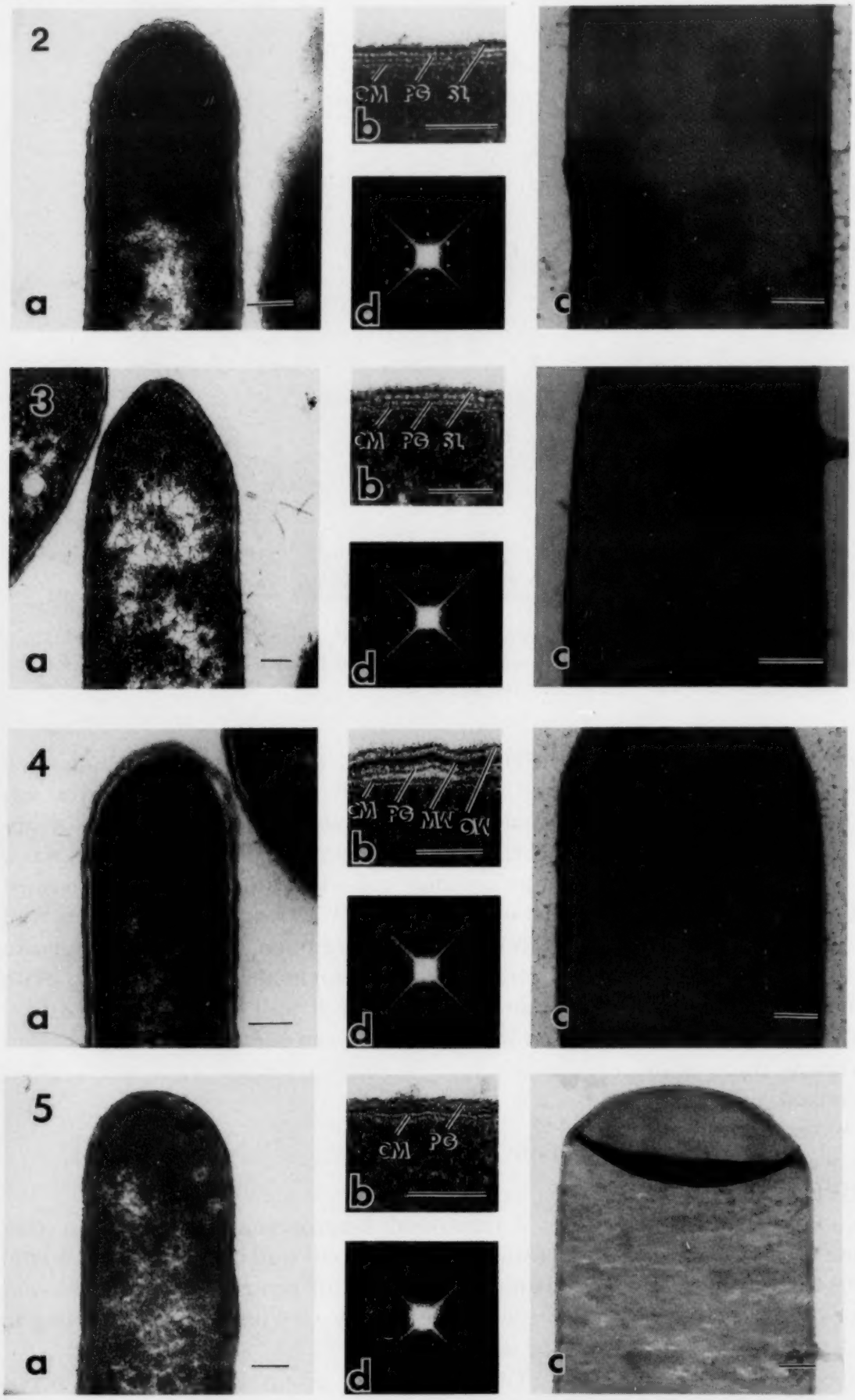

Fig. 2–5. *Bacillus brevis* HPD31 (Fig. 2); HPD52 (Fig. 3), HP033 (Fig. 4) and HP034 (Fig. 5) in ultrathin sectioned preparation of intact cells (a, b) and negatively stained preparations of cell envelopes (c) with the respective optical diffraction patterns (d). All S-layers observed were of hexagonal symmetry as illustrated in the Moiré-patterns of the negative stains (Fig. 2–4c). CM: cytoplasmic membrane, PG: peptidoglycan, SL: Surface layer, OW: outer wall, MW: middle wall. Bars in a and c represent 200 nm, bars in b 50 nm.

walls were analyzed by SDS-PAGE (Fig. 1A). This group with the strains HP019, HP020, HP034, HPD61 and HPD71 has therefore the most common type of Gram-positive cell wall [8]. This has not been described yet with wild-type strains of protein-producing bacteria.

The cell wall of group II bacteria consists of a thin peptidoglycan-layer and an outermost S-layer. A regular staining pattern in negatively contrasted cell wall preparations (Fig. 2, 3c) and the characteristic two-layered cell wall in ultrathin sections have been found with the strains HPD31 and HPD52 (Fig. 2, 3). Therefore, group II strains have the same morphological characteristics as most S-layer-carrying bacteria [9]. However, both strains showed more than one single major protein band in electropherograms of cell wall extracts (Fig. 1A). This observation has frequently been reported with S-layer-carrying bacteria as in the case of *Bacillus stearothermophilus* NRS 2004/3a [10]. The cell walls of group II bacteria, though representing a known structure, have not been described yet with protein-producing bacteria. We introduced group III for *B. brevis* strains with the same cell wall characteristics as *B. brevis* 47 [2,3]. These are, as illustrated with strain HP033, two major proteins in the cell wall (Fig. 1A), a regular staining pattern in the respective negative stainings (Fig. 4c) and two subunit type layers outside the thin peptidoglycan layer in ultrathin sections (Fig. 4a, b). A similar multi-layered cell wall structure has also been described for non-protein-producing bacteria, such as *Bacillus* sp., strain C.I.P. 76-111 [11].

The observation that the 135 kDa protein of strain HPD31, the 115 kDa protein of strain HPD52 and the 110 kDa protein of strain HP033 were immunologically related to the strain 47 MW-protein only (Fig. 1B) and showed no relation to the strain 47 OW-protein was an unexpected result. Accordingly, not the S-layers which constitute the outermost cell wall-layer are similar to each other, but the S-layers which are located adjacent to the peptidoglycan-layer are related to each other whether they are the outermost cell wall layer (group II) or not (group III). These findings are interpreted in terms of conservation, i.e. the S-layer adjacent to the peptidoglycan-layer would be less variable than additional S-layers.

For the group III organism *B. brevis* 47, the sequences of both the OWP and MWP have been determined [3,12]. As expected from immunological evidence, they showed no significant homology. However, to find out the regions characteristics for the immunologically related kinds of S-layers, more data on S-layer DNA-sequences have to be provided. Therefore, experiments at genetic level with further *B. brevis* strains, especially HP033, are currently undertaken.

REFERENCES

[1] Udaka, S. (1976) Agric. Biol. Chem. 40, 523–528.

[2] Tsuboi, A., Tsukagoshi, N. and Udaka, S. (1982) J. Bacteriol. 151, 1485–1497.

[3] Tsuboi, A., Uchihi, R., Tabata, R., Takahashi, Y., Hashiba, H., Sasaki, T., Yamamgata, H., Tsukagoshi, N. and Udaka, S. (1986) J. Bacteriol. 168, 365–373.

[4] Ohmizu, H., Sasaki, T., Tsukagoshi, N., Udaka, S., Kaneda, N. and Yagi, K. (1983) J. Biochem. 94, 1077–1084.

[5] Yamada, H., Tsukagoshi, N. and Udaka, S. (1981) J. Bacteriol. 148, 322–332.

[6] Laemmli, U.K. (1970) Nature (London) 227, 680–685.

[7] Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.

[8] Shockman, G.D. and Barrett, J.F. (1983) Ann. Rev. Microbiol. 37, 501–527.

[9] Sleytr, U.B. and Messner, P. (1983) Ann. Rev. Microbiol. 37, 311–339.

[10] Küpcü, Z., März, L., Messner, P. and Sleytr, U.B. (1984) FEBS Lett. 173, 185–190.

[11] Leduc, M., Rousseau, M. and Heijenoort, J. (1977) Eur. J. Biochem. 80, 153–163.

[12] A. Tsuboi, Uchihi, R., Adachi, T., Sasaki, T., Hayakawa, S., Yamagata, H., Tsukagoshi, N. and Udaka, S. (1988) J. Bacteriol. 170, 935–945.

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Rapid small-scale DNA isolation from filamentous cyanobacteria

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1. SUMMARY

A rapid small-scale DNA isolation procedure is described for the filamentous cyanobacteria, which yields enough chromosomal and plasmid DNA for restriction endonuclease digestions, Southern hybridizations, and electroelution from gels for further manipulation. DNA from seven strains of cyanobacteria were isolated and analyzed on agarose gels.

2. INTRODUCTION

Cyanobacteria are Gram-negative, photosynthetic prokaryotes, with some species capable of fixing atmospheric dinitrogen. There has been increasing interest in the regulation of these and other processes in cyanobacteria at the molecular level. Methods are being developed to transfer cloned DNA to cells [1–7] so that regulation can be studied using molecular genetics.

Although methods for isolating DNA from cyanobacteria have been published [8–11], many are time-consuming and require large amounts of material. Separate isolations for both plasmid and chromosomal DNA are used, as well as different procedures depending on the plasmid size. There are many instances where it is necessary to rapidly obtain DNA for various screenings or other manipulations. In this study, we have isolated both plasmid and chromosomal DNA from a number of filamentous cyanobacteria using a simple lysozyme method which yields chromosomal DNA and plasmids rapidly in one preparation. This method is useful for examining plasmid profiles, for Southern blot hybridizations, and for use in cloning of both plasmids and chromosomal DNA.

3. MATERIALS AND METHODS

3.1. Cyanobacteria strains and growth conditions

Plectonema strains, *P. boryanum* 594, 581, 1566, and *P.* sp. 1541 were purchased from the University of Texas Culture Collection (UTEX). *Oscillatoria tenuis* 1566 and *Phormidium autumunale* 1580 also were obtained from UTEX. Two cultures were obtained from the American Type Culture Collection, the LPP strains 27913 and 29117. Cells were grown in Erlenmeyer flasks containing 50 ml BG-11 liquid medium [12] at 30°C in an orbital shaker-incubator under fluorescent lights.

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3.2. DNA isolation

Cultures were grown until mid to late log phase and centrifuged for 10 min at 10000 rpm. Pellets were washed in 50 mM Tris-HCl (pH 8.0)–5 mM EDTA–50 mM NaCl and resuspended in 2 ml 50 mM Tris-HCl–50 mM EDTA, pH 8.0. Lysozyme (45 mg/ml stock resuspended in ^2H_2O) was added (1 ml) to give a final concentration of 15 mg/ml. Cells were incubated at 37°C for 1 hour. An equal volume of 30 mM Tris–10 mM EDTA–2% sarkosyl then was added and the tubes were mixed gently. Pretreated Ribonuclease A was immediately added at a final concentration of 10 μg/ml and the lysates were incubated at 37°C for 45 minutes. This was followed by two phenol, two chloroform, and two ether extractions. DNA was precipitated in 2.5 volumes of ethanol in a dry-ice ethanol bath for 15 minutes. Precipitation can be done overnight at −20°C. DNA then was pelleted at 12000 rpm for 15 min in a microfuge. The pellets were washed in 70% ethanol, centrifuged again, and resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. Absorbance ratios at $A_{260/280}$ were in range of 1.7–1.9. Up to 100 μg of DNA (or more) were obtained from each isolation.

Fig. 2. Agarose gel (0.8%) of total *Phormidium autumunale* 1580 DNA. Intact chromosomal DNA and two plasmids (arrows), 16.7 kb and 2.7 kb, are clearly visible.

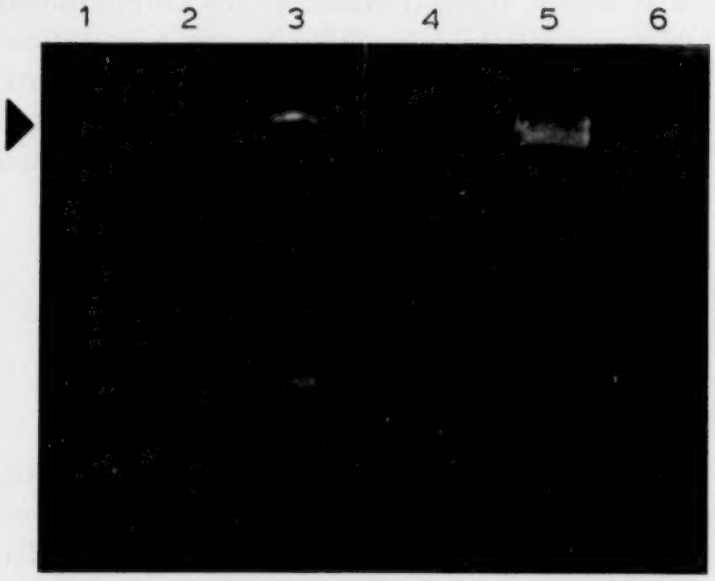

Fig. 1. DNA (1/200 of total sample) from six isolations were run on a 0.8% mini gel. Lane 1, PB581; Lane 2 PB594; Lane 3, OT1566; Lane 4, P1541; Lane 5, 29117; Lane 6, 27913. Both 27913 and 29117 do not contain plasmids. *Plectonema* 581 and 594 contain a 12.9 kb and a 1.6 kb plasmid, although only the small plasmid is seen since the large plasmid is in very low copy number. OT1566 contains two plasmids and P1541 contains at least three plasmids.

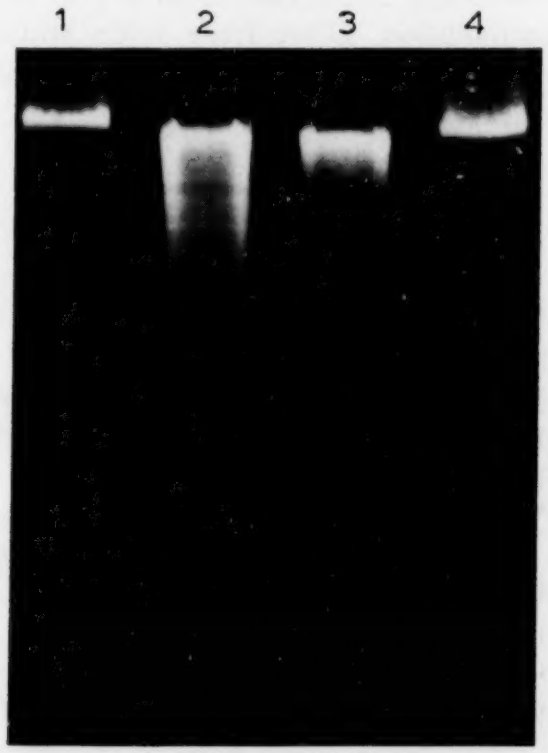

Fig. 3. Mini gel of total DNA from PB581 (Lane 1, uncut; Lane 2, *Cla*I digested), and PB594 (Lane 3, *Cla*I digested; Lane 4, uncut). Plasmid DNA was not visible due to the small amount of DNA loaded onto the gel. The gel has been overstained.

3.3. Restriction endonuclease digestions

Restriction endonucleases were purchased from Promega Biotec and New England Biolabs, Inc. and were used according to manufacturers' specifications.

4. RESULTS AND DISCUSSION

We have obtained high-molecular weight chromosomal DNA from cells that can be lysed with lysozyme using a small-scale, rapid isolation procedure (Fig. 1, Lanes 1–6). Although plasmids are present in very low copy number, they were visualized on gels (Fig. 1, Lanes 1–4; Fig. 2). These profiles have been confirmed using a small-scale plasmid purification procedure [13]. Chromosomal DNA was observed as a discrete, high-molecular weight band; however, upon digestion with a variety of restriction enzymes, the chromosomal DNA band was readily digested into smaller fragments as demonstrated by smearing (Fig. 3, Lanes 2–3; Fig. 4, Lanes 2–4). Although cyanobacterial plasmids are resistant to cleavage by many restriction enzymes, digested plasmid DNAs were visualized as discrete bands (Fig. 4).

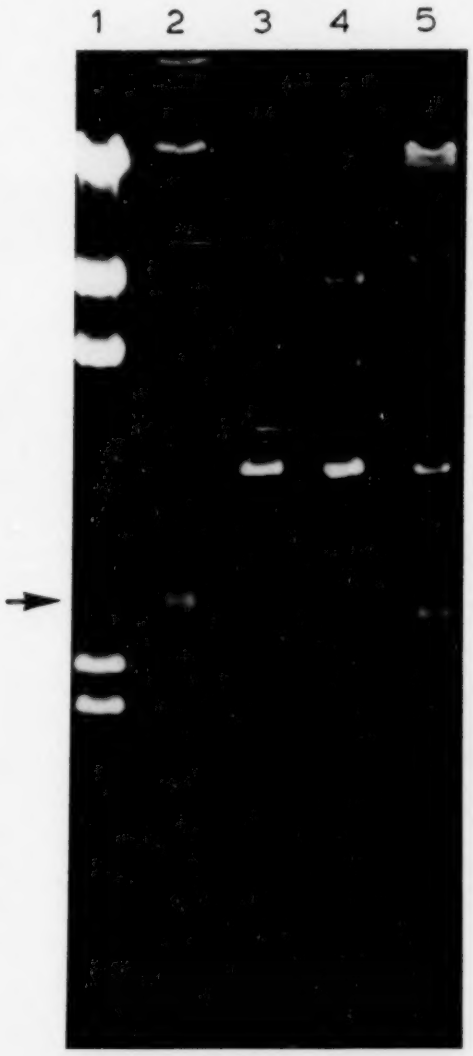

Fig. 4. Agarose gel (0.8%) of DNA isolated from *Plectonema* 1541. Lane 1, lambda DNA cut with *Hin*dIII; Lane 2, uncut total DNA; Lane 3, *Cla*I; Lane 4, *Hpa*I; Lane 5, *Fok*I. A small plasmid is visualized in Lanes 2 and 5 (arrow). Larger plasmids are barely detectable in the gel (Lane 2). Digested plasmids are seen in Lanes 3–5 as discrete bands.

This method has the advantage of being rapid and yielding enough DNA (both plasmid and chromosomal) for manipulations such as Southern blot hybridizations, electroelution, cloning, and determination of plasmid profiles, etc. Chromosomal DNA may mask plasmid profiles due to shearing, especially if plasmids are low in copy number, or there is broadening of the DNA band in the gel [10,14,15]. Other methods such as spooling and ultracentrifugation also produce some breakage (unpublished observations). In the method described here, plasmids at least 17 kb in size, are not obscured since the procedure is gentle enough so that chromosomal and plasmid DNAs are not sheared.

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REFERENCES

[1] Astier, C. and Espardellier, F. (1976) C. R. Hebd. Seances Acad. Sci. Ser. D 282, 795–797.
[2] Stevens, S.E. and Porter, R.D. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 6052–6056.
[6] Kuhlemeier, C.J., Borrias, W.E., van den Hondel, C.A.M.J.J. and van Arkel, G.A. (1981) Mol. Gen. Genet. 184, 249–254.
[4] Sherman, L.A. and van de Putte, P. (1982) J. Bacteriol. 150, 410–413.
[5] Chauvat, F., de Vries, L., van der Ende, A. and van Arkel, G.A. (1986) Mol. Gen. Genet. 204, 85–191.
[6] Flores, E. and Wolk, C.P. (1985) J. Bacteriol. 162, 1339–1341.

[7] Wolk, C.P., Vonshak, A., Kehoe, P. and Elhai, J. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 1561–1565.
[8] van den Hondel, C.A.M.J.J., Keegstra, W.E., Borrias, W.E. and van Arkel, G.A. (1979) Plasmid 2, 323–333.
[9] Barnum, S.R. and Gendel, S.M. (1985) FEMS Microbiol. Lett. 29, 339–442.
[10] Potts, M. (1984) Focus 6, 4–6.
[11] Lambert, G.R. and Carr, N.G. (1982) Arch. Microbiol. 133, 122–125.
[12] Allen, M.M. (1968) J. Phycol. 4, 1–4.
[13] Felkner, R.H. and Barnum, S.R. (1988) Curr. Microbiol. 17, 37–41.
[14] Engwall, K.S. and Gendel, S.M. (1985) FEMS Microbiol. Lett. 26, 337–340.
[15] Franche, C. and Reynaud, P.A. (1986) Ann. Inst. Pasteur Microbiol. 137A, 179–197.

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High frequency transformation of *Bordetella* by electroporation

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Key words: *Bordetella*; Transformation; Electroporation; Electric field pulses

1. SUMMARY

A simple and efficient system is described for the transformation of *Bordetella* species with a plasmid of the P incompatibility group. The parameters tested to obtain the highest frequency of transformation were strain, initial field strength, number of pulses and DNA and cell concentration. The most important variable was found to be the initial field strength and at 25 kV/cm transformants could be obtained at a frequency of $> 10^6/\mu g$.

2. INTRODUCTION

Exposure of cells to brief pulses of electric current results in a reversible permeabilisation of the biomembranes and has been termed electroporation. Electroporation has been used to introduce DNA into a variety of cell types. Techniques for the transformation of mammalian [1–3], plant [4], bacterial [5–8], yeast cells [9] and parasites [10,11] have been previously described. We have tested the applicability of electroporation to the transformation of the important bacterial pathogen *Bordetella pertussis* and the closely related *B. parapertussis*. Whooping cough (pertussis) is a disease of the upper respiratory tract caused by a localized infection by *B. pertussis*. The absence of a technique for high-frequency transformation has limited the genetic manipulation of this organism. Weiss and Falkow [12] described that *B. pertussis* cells cannot be transformed by classical methods such as calcium chloride treatment but that a cold shock or freezing of the cells results in transformation by plasmids of the P and W incompatibility groups. The transformation frequency obtained was less than $10^3/\mu g$ of DNA however and a restriction system in *B. pertussis* prevents the introduction of plasmids containing the *Hind*III recognition sequence. More recently a modified form of this technique has been used to transform *B. bronchiseptica* at a frequency of $10^4/\mu g$ of DNA [13]. During our studies on the genetics of *B. pertussis* we have used electroporation to transform this bacterium. In this report we describe a systematic investigation of parameters influencing the frequency of transformation of *B. pertussis* by electroporation and provide a simple and efficient technique that routinely yields $> 10^6$ transformants per μg of plasmid DNA.

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Abbreviations: kV, kilovolts; cfu, colony forming units.

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3. MATERIALS AND METHODS

3.1. Bacterial strains , plasmids, media and buffers

B. pertussis GRZ2 is a spontaneous streptomycin resistant isolate of the Connaught production strain 10536. *B. parapertussis* ATCC 15311 was obtained from the American Type Culture Collection, Rockville, Maryland. Plasmid pRK404ΔMCS is a derivative of the broad host range cloning vector pRK404 [14] in which the multiple cloning site has been deleted. Plasmid DNA was isolated from *Escherichia coli* JM109 (*rec*A1, *end*Al, *gyr*96, *thi*, *hsd*R17, *sup*E44, *rel*Al, Δ(*lac-pro*AB), [F^-, *tra*D36, *pro*AB$^+$, *lac*I^q, ZΔM15] as described by Ish-Horowicz and Burke [15] and chromosomal DNA from *B. pertussis* transformants by the method of Yacoob and Zealey [16]. Electroporation buffer was 300 mM sucrose, 1 mM $MgCl_2$, 7 mM potassium phosphate buffer pH 7.2, and had a specific resistivity of 1250 ohm · cm. TE buffer was 10 mM Tris · HCl, 1 mM EDTA pH 7.5.

Liquid cultures of *B. pertussis* were grown in modified CL medium [17] and plate cultures on Bordet Gengou medium (Gibco) with 20% fresh, defibrinated sheep blood (Bockneck). Streptomycin and tetracycline were added to medium at concentrations of 100 μg/ml and 10 μg/ml respectively.

3.2. Transformation by electroporation

B. pertussis cells were grown in 100 ml of liquid medium to a cell density of about 10^9 cells/ml, harvested in a clinical centrifuge (4000 × *g*, 15 min, 20°C), washed in 25 ml of electroporation buffer and resuspended in 10 ml of the same. Plasmid DNA (typically 10–50 ng in a volume of 1–5 μl of TE) was added to 500 μl of the cell suspension and the mixture incubated on ice for 10 min. The cells were subjected to exponential decay voltage pulses with a BTX Transfector 100 using a 0.8 mm gap, 472 cuvette electrode (Biotechnologies and Experimental Research, San Diego, CA) at room temperature. Field strengths were determined as described by the manufacturer. Three ml of media were added and the cells incubated with shaking at 37°C for 60 min. The cells were harvested by centrifugation in a microfuge for 15 s at room temperature, resuspended in 100 μl of media, spread onto a Bordet Gengou plate with antibiotic selection and incubated for 2–5 days at 37°C.

4. RESULTS

4.1. Factors affecting transformation efficiency

Factors affecting transformation efficiency were investigated. Transformation efficiency was found to be essentially independent of cell concentration over the range of 10^8 to 10^{10} cells/ml and showed a linear response to DNA concentrations between 1 ng and 1 μg/ml. In contrast to this, transformation efficiency was found to be directly proportional to the initial electric field strength (Fig. 1). Thus few transformants (1–10/μg DNA) were obtained at field strengths below 8 kV/cm and the greatest efficiency ($> 10^6$/μg DNA) was obtained at about 25 kV/cm. This value is the highest field strength that can be generated by the BTX 100 Transfector under these experimental conditions. Transformation frequency was therefore determined after subjecting the cells to between 1 and 5 sequential discharges at an initial field strength of 25 kV/cm. Multiple discharges did not result in an increase in the number of transformants obtained however (Fig. 1).

The conditions for optimal transformation of *B. parapertussis* were also investigated using the BTX transfector and were found to be identical to those for *B. pertussis*. Weiss and Falkow [12] reported a restriction system in *B. pertussis* Tohama III strain that limits the introduction of plasmids containing the *Hind*III recognition sequence. The Connaught production strain appears to have a similar restriction system as shown by the reduced transformation efficiency by plasmid pRK404 which has a single *Hind*III site (Fig. 1). This reduction presumably indicates that whilst many of the plasmid molecules introduced are restricted, some are modified by the host and are therefore maintained in the cell.

No transformants were obtained if DNA was omitted from the electroporation mixture or if the cells were not subjected to an electrical discharge.

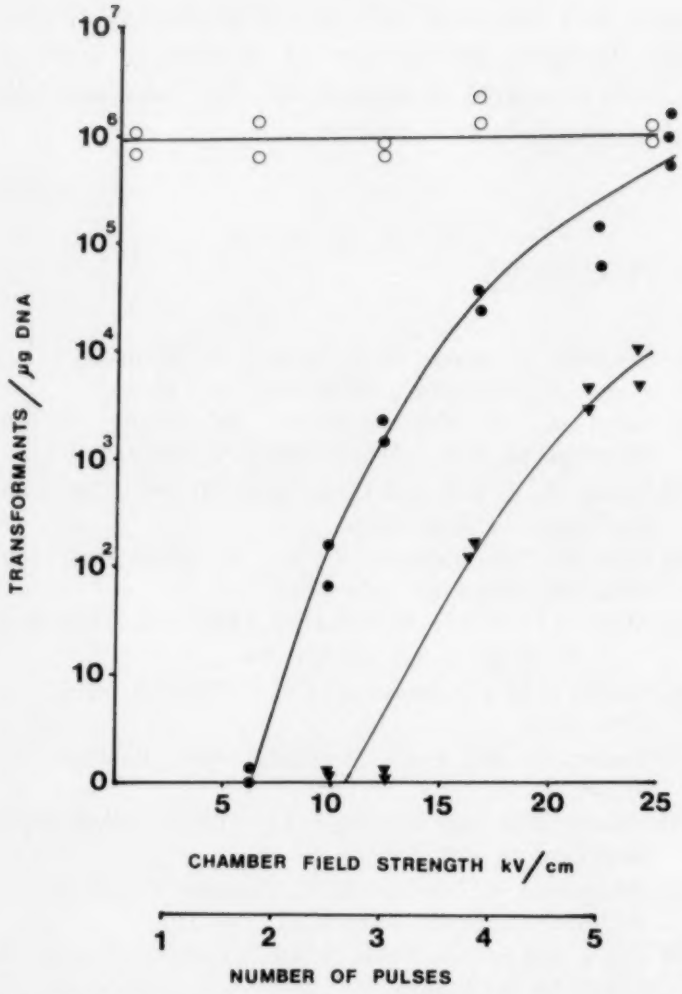

Fig. 1. Efficiency of transformation of *Bordetella pertussis* by electroporation as a function of initial electric field strength (●, pRK404ΔMCS; ▼, pRK404) and number of pulses (○) with pRK404ΔMCS plasmid DNA. For multiple pulses cells were incubated on ice for 10 min between discharges. The pulse length in these experiments was about 40 μs and is defined as the time taken for the pulse to decay to $1/e$ of the applied voltage where e is the natural logarithm of that voltage. Thus, the pulse is typically maintained at the indicated field strength for about 1/3 of its length (i.e. about 15 μs).

4.2. *Effect of electroporation upon cell survival*

In yeast, mammalian and some bacterial systems a relationship could be established between the frequency of transformation by electroporation and cell killing [6–10]. Furthermore several species of bacteria have been shown to be killed by electrical discharges under some conditions [18]. *B. pertussis* however shows a similar survival response to electrical discharges as the Gram-negative bacterium *Campylobacter jejuni* [5] in that there was no statistically significant reduction in cell viability even at the highest field strenghts applied (Table 1).

Table 1

Survival of *Bordetella pertussis* GRZ2 after electroporation.

Initial electric field strength (kV/cm)	Viable cells (cfu/ml × 10^{10})
0	1.7 ± 0.27
9.5	1.4 ± 0.26
12.8	2.0 ± 0.29
16.0	1.5 ± 0.40
19.1	1.4 ± 0.36
22.3	1.6 ± 0.39
25.5	1.2 ± 0.47

Cells were subjected to electroporation in the absence of DNA, diluted and plated onto Bordet Gengou plates without antibiotics for enumeration. Values reported are means ± standard errors for triplicate samples at 95% confidence limits.

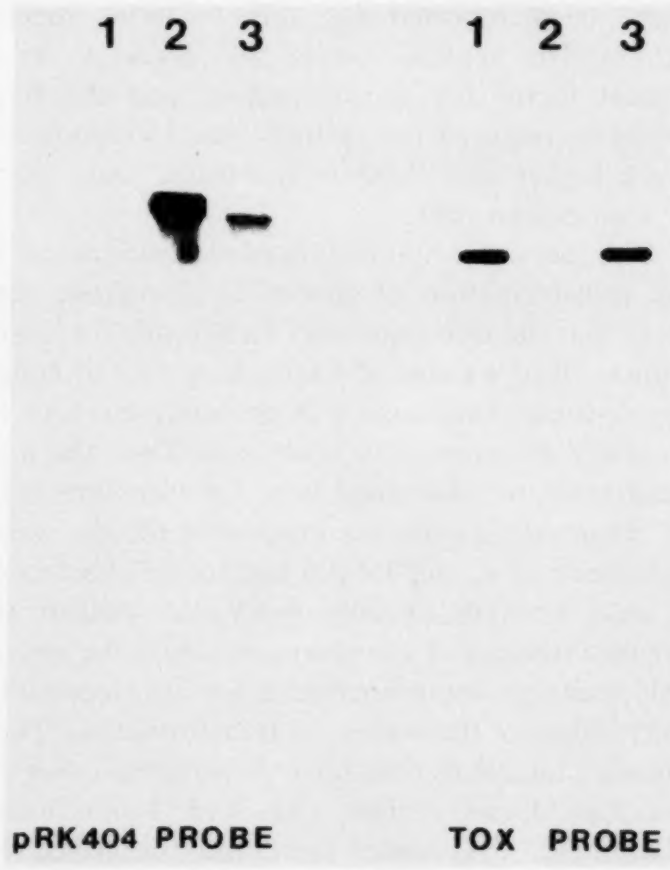

Fig. 2. Southern blot hybridization of total cellular DNA demonstrating intact plasmid in transformants of *Bordetella pertussis* obtained by electroporation. Lane 1: untransformed *B. pertussis* 2: plasmid pRK404ΔMCS, 3: *B. pertussis* [pRK404ΔMCS] transformants. DNA samples were restricted with *Eco*RI and probed with nick translated pRK404ΔMCS plasmid or a *B. pertussis* toxin gene specific probe as indicated. Hybridization and nick translation were performed as described by Maniatis et al. [20].

4.3. Analysis of transformants

Antibiotic resistant colonies obtained following electroporation were confirmed as *B. pertussis* transformants by detection of the *B. pertussis* specific antigen pertussis toxin in culture supernatants [19] and by Southern blot analysis of total cellular DNA using plasmid or *B. pertussis* toxin gene specific probes as shown in Fig. 2.

5. DISCUSSION

We have shown that high-voltage electroporation provides a simple means for the high-frequency transformation of *Bordetella* species which are difficult to transform by conventional methods. The frequency of transformation obtained is 100–10 000 fold higher than has previously been reported for this bacterial species [12,13]. The applied voltage was found to be a critical factor for transformation and the field strengths required for optimal transformation are much higher than those to transform yeast, plant or mammalian cells.

The successful application of electroporation to the transformation of species of *Bordetella* suggests that the technique may be suitable for transformation of a range of bacterial species although the optimal conditions will probably have to be carefully determined in each case. Thus the high field strengths described here for transformation of *Bordetella* species are unsuitable for the transformation of *E. coli* JM109 and for this bacterium a field strength of only 6 kV/cm yielded the greatest number of transformants. Also the electrical discharge equipment used for electroporation may influence the success of transformation. Thus, we were unable to transform *B. pertussis* using the Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) under conditions described for efficient transformation of the pathogenic Gram-negative bacterium *C. jejuni* [5]. Variables affecting transformation frequency may include the shape and duration of the pulse, initial electric field strenght, the number of discharges applied, the physiological condition of the cells and the electroporation buffer used.

REFERENCES

[1] Bischoff, R., Eisert, R.M., Schedl, I., Vienken, J. and Zimmerman, U. (1982). FEBS Lett. 147, 64–68.

[2] Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) EMBO J. 1, 841–845.

[3] Potter, H., Weir, L. and Leder, P. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 7161–7165.

[4] Ou-Lee, T-M., Turgeon, R., Wu, R. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6815–6819.

[5] Miller, J.F., Dower, W.J. and Tompkins, L.S. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 856–860.

[6] Calvin, N.M and Hanawalt, P.C. (1988) J. Bacteriol. 170, 2796–2801.

[7] Fiedler, S. and Wirth, R. (1988) Anal. Biochem. 170, 38–44.

[8] Chassy, B.M. and Flickinger J.L. (1987). FEMS Microbiol. Lett. 44, 173–177.

[9] Hashimoto, H., Morikawa, H., Yamada, Y. and Kimura. A. (1985) Microbiol. Biotech. 21, 336–339.

[10] Eid, J. and Sollner-Webb, B. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 7812–7816.

[11] Gibson, W.C., White, T.C., Laird, P.W. and Borst, P. (1987) EMBO J. 6, 2457–2461.

[12] Weiss, A.A. and Falkow, S. (1982) J. Bacteriol. 152, 549–552.

[13] Lax, A.J. (1987) Nucleic Acids Res. 15, 856.

[14] Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X-W., Finlay, D., Guiney, D. and Helinski, D. (1985) Plasmid 13, 149–153.

[15] Ish-Horowicz, D. and Burke, J.F. (1981) Nucleic Acids Res. 9, 2989–2998.

[16] Yacoob, R.K. and Zealey, G.R. (1988) Nucleic Acids Res. 16, 1639.

[17] Imaizumi, A., Suzuki, Y., Ono, S., Sato, H. and Sato, Y. (1983) Infect. Immun. 41, 1138–1143.

[18] Hulsheger, H., Potel, J. and Nieman, E.-G. (1983) Radiat. Environ Biophys. 22, 149–162.

[19] Gillenius, P., Jaatmaa, E., Askelof, P., Granstrom, M. and Tiru, M. (1985) J. Biol. Stand. 13, 61–66.

[20] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, Cold Spring Laboratory, Cold Spring Harbor, NY.

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VOL. 56 No. 1 CONTENTS NOVEMBER 1988

Research Letters

Methods for the identification and analysis of L-altrose in bacterial polysaccharides (FEM 03330)
R.J. Stack and L.D. Ericsson (U.S.A.) 1

Identification of a methylase gene for erythromycin resistance within the sequence of a spontaneously deleting fragment of *Corynebacterium diphtheriae* plasmid pNG2 (FEM 03331)
T.M. Serwold-Davis and N.B. Groman (U.S.A.) 7

Spherical and rod shaped protoplasts from β-lactam antibiotic treated cultures of *Bacillus subtilis* (FEM 03332)
G. Wright and H.J. Rogers (U.K.) 15

Mapping and disruption of the *cybB* gene coding for cytochrome b_{561} in *Escherichia coli* (FEM 03333)
I. Yamato, H. Nakamura, H. Murakami and Y. Anraku (Japan) 21

Thymidine phosphorylase and uridine phosphorylase of *Lactobacillus casei* (FEM 03334)
Y. Avraham, J. Yashphe and N. Grossowicz (Israel) 29

An *Escherichia coli* enterobactin cluster gene with sequence homology to *trpE* and *pabB* (FEM 03335)
M.F. Elkins and C.F. Earhart (U.S.A.) 35

Effects of penicillin on the synthesis of membrane proteins of *Chlamydia trachomatis* LGV2 serotype (FEM 03336)
R. Cevenini, M. Donati and M. La Placa (Italy) 41

Exocellular products from *Bifidobacterium adolescentis* as immunomodifiers in the lymphoproliferative responses of mouse splenocytes (FEM 03337)
E. Gómez, M.M. Melgar, G. Pérez Silva, A. Portolés and I. Gil (Spain) 47

Cloning of a maltogenic alpha-amylase from *Bacillus stearothermophilus* (FEM 03338)
B. Diderichsen and L. Christiansen (Denmark) 53

Behaviour of Nif$^-$ mutants of *Rhodobacter capsulatus* in photosynthetic, ammonia-limited chemostat cultures (FEM 03339)
J.C. Willison and B. Lac (France) 61

Improvements in the transformation of *Bacillus subtilis* protoplasts with plasmid DNA (FEM 03340)
C.P. Rubinstein, L.C. Moratinos, O.A. Coso and C. Sánchez-Rivas (Argentina) 67

The effect of thiols and the Ca^{2+} ionophore A23817 on growth and antibiotic production in *Cephalosporium acremonium* (FEM 03341)
M.J.C. Crabbe (U.K.) 71

The properties and structure of the membrane ATPase from *Vibrio alginolyticus* (FEM 03342)
O.Y. Dmitriev and B.V. Chernyak (U.S.S.R.) 79

Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation (FEM 03343)
J.D. Oultram, M. Loughlin, T.-J. Swinfield, J.K. Brehm, D.E. Thompson and N.P. Minton (U.K.) 83

On the presence of calmodulin-like protein in mycobacteria (FEM 03344)
A.M.S. Falah, R. Bhatnagar, N. Bhatnagar, Y. Singh, G.S. Sidhu, P.S. Murthy and T.A. Venkitasubramanian (India) 89

Transformation of group A streptococci by electroporation (FEM 03345)
A. Suvorov, J. Kok and G. Venema (U.S.S.R., The Netherlands) 95

Effect of growth conditions on the rate of loss of the plasmid pAT153 from continuous cultures of *Escherichia coli* HB101 (FEM 03346)
L.C.K. Chew, W.C.A. Tacon and J.A. Cole (U.K.) 101

Effect of nicking on Shiga-like toxin I of enterohaemorrhagic *Escherichia coli* (FEM 03347)
U. Kongmuang, T. Honda and T. Miwatani (Japan) 105

Haemagglutinating activity of *Campylobacter pylori* (FEM 03348)
J. Huang, C.J. Smyth, N.P. Kennedy, J.P. Arbuthnott and P.W. Napoleon Keeling (Republic of Ireland) 109

Comparative study on the cell wall structure of protein-producing *Bacillus brevis* (FEM 03349)
K. Gruber, H. Tanahashi, A. Tsuboi, N. Tsukagoshi and S. Udaka (Japan) 113

Rapid small-scale DNA isolation from filamentous cyanobacteria (FEM 03350)
J.A. Smoker and S.R. Barnum (U.S.A.) 119

High frequency transformation of *Bordetella* by electroporation (FEM 03351)
G. Zealey, M. Dion, S. Loosmore, R. Yacoob and M. Klein (Canada) 123

Cited in: Biological Abstracts; Chemical Abstracts; Current Contents (Life Sciences); Excerpta Medica; Pascal